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THE DETERMINATION OF MAGNESIUM AND CALCIUM IN SERUM OR PLASMA WITH ETHYLENEDIAMINETETRA-ACETIC ACID (EDTA)

By

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INTRODUCTION

The existing methods for the determination of calcium and magnesium in serum suffer from a number of drawbacks. The common methods for calcium involve precipitation of calcium as oxalate, washing the precipitate and determining the oxalate ion with potassium permanganate either titrimetrically or colorimetrically. Recently other methods have been described in which the calcium is titrated with EDTA using murexide (ammonium purpurate) as an indicator, (Sobel and Hanok, 1951; Buckley, Gibson and Bortolotte, 1951; Holtz and Seekles, 1952; Elliot, 1952; Mulligan and Jennings, 1953). Most of these methods were devised for human sera which normally contain little or no carotenoid. Bovine serum or plasma contains large amounts of carotenoid and the observation of the end point is rendered difficult unless the colours are compared spectroscopically, (Holtz and Seekles, 1952). Wilson (1953) has discussed the use of these methods in determining calcium and magnesium in urine.

Methods for the determination of magnesium in serum fall into three classes. (1) After removal of the calcium as oxalate the magnesium is precipitated as magnesium ammonium phosphate, washed and the phosphate converted to a coloured complex such as molybdenum blue (Briggs, 1922; Youngburg and Youngburg, 1930) or molybdovanadate complex (Simonsen, Westover and Westman, 1947. (2) Direct formation of a Titan yellow lake using a trichloracetic acid filtrate of serum, (Garner, 1946; Heagy, 1948; Orange and Rhein, 1951). (3) By titration with EDTA at pH 10 in an ammoniacal buffer, (Sobel and Hanok, 1951 ; Buckley, *et al.*; 1951). In these methods the total concentration of calcium and magnesium is determined by using Eriochrome Schwarz as an indicator, the calcium by using murexide as an indicator and the magnesium by difference.

In the author's opinion all these methods for determining magnesium suffer from disadvantages. Those which involve the formation of a coloured phosphate complex are time consuming as the precipitates must be washed at least three times to remove excess of precipitating reagent: moreover the ratio of phosphate to magnesium in magnesium ammonium phosphate is somewhat dependent on the ambient conditions.

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The biggest disadvantage of the Titan Yellow method is the low slope of the "optical density" versus "concentration" curve. On the other hand the titration of calcium and magnesium in bovine serum or plasma with EDTA is rendered difficult by the presence of carotenoids which obscure the end points of the indicators, and the magnesium, is, of necessity, determined by difference.

The method described in this paper is an attempt to overcome some of these difficulties. Briefly, the calcium is first precipitated as calcium oxalate and the magnesium in the supernatant solution is then precipitated as magnesium ammonium phosphate. The precipitates are dissolved in nitric acid and titrated with 0.002 M. EDTA in the presence of potassium cyanide, in an ammoniacal buffer of pH 10 using Solochrome Black WDFA as the indicator. These procedures are not so quick as the direct methods but the calcium and magnesium are separated as calcium oxalate and magnesium ammonium phosphate respectively, and the calcium or magnesium titrated separately with EDTA.

Calcium ions will form a reversible coloured complex with Solochrome Black as an indicator but a sharper end point is obtained if the magnesium complex of EDTA is also present. For this reason the magnesium is titrated first and then added to the calcium immediately before it is titrated. The reactions involved are rather slow, but warming the solutions to between 60 to 80°C speeds the reactions and sharpens the end point. As it is the magnesium ion which is titrated neither the presence of traces of phosphate from the precipitating reagent nor an increased phosphate to magnesium ratio in the precipitate is of any importance, hence there is no need to wash the precipitates. For similar reasons the calcium oxalate precipitates do not require washing.

Buckley, *et al.* (1953) in their direct method, recommend the use of potassium cyanide to prevent heavy metal interference, but in this method potassium cyanide is used to help to sharpen the end point.

METHODS

Reagents

EDTA. Disodium ethylenediaminetetra-acetic acid .002*M*. Dissolve 0.7445 g. in water, adjust to pH 10 with N.NaOH and make up to 1 litre.

Buffer solution. Dissolve 67.5 g. of ammonium chloride A.R. in 570 ml. of strong ammonia A.R. (SG. 0.880) and make up to 1 litre. (Heald, Cootes and Edwards, 1950).

Indicator solution. Dissolve 4.5 g. hydroxylamine hydrochloride and 0.2 g. Solochrome Black WDFA (ICI Ltd.) in 100 ml. absolute alcohol. This solution keeps well in the refrigerator. (Heald, *et al.* 1950).

Ammonium Phosphate, 5 per cent; Potassium Cyanide, 0.7 Molar; Ammonium Oxalate, 4 per cent; Standard solution containing 10 mg. calcium and 2.0 mg. magnesium per 100 ml.

All the chemicals used should be of A.R. Grade and only glass distilled water should be used.

Procedure

The method falls naturally into two phases, (1) the precipitation of the calcium as calcium oxalate and of magnesium as magnesium ammonium phosphate; (2) the titration of the calcium and magnesium in the precipitates with 0.002M. EDTA.

Mix 2 ml. of plasma or serum with 2 ml. glass distilled water in a centrifuge tube, add 2 ml. 4 per cent ammonium oxalate and stand for 45 mins. Centrifuge at 2500 to 3000 r.p.m. for 15 mins. This procedure precipitates the calcium as oxalate; the supernatant is poured off and the tube allowed to drain. Five ml. of the supernatant is then added to a freshly prepared mixture of 0.9 ml. 5 per cent ammonium phosphate and 0.9 ml. conc. ammonia and allowed to stand in the refrigerator for at least 4 hours, but preferably overnight. The precipitate of magnesium ammonium phosphate is centrifuged down, the supernatant discarded and the tube drained on filter paper. The precipitates are dissolved in 50 per cent nitric acid, 3 or 4 drops being required to dissolve the calcium oxalate and 1 drop the precipitate of magnesium ammonium phosphate.

Titrations of the calcium and magnesium are carried out at a temperature of at least 60°C. The tubes containing the dissolved precipitates are stood in a water bath at about 80°C. One ml. of 0.7 M. KCN and 2 ml. of buffer are added to the tube containing the magnesium. When the evolution of ammonia commences, 2 drops of indicator are added and the EDTA solution is run in slowly from a 5 ml. burette calibrated to 0.01 ml. The end point is when the indicator changes from wine red to a pale blue. After reading the burette the titrated magnesium solution is poured into the calcium tube, another 1 ml. of buffer added and the titration repeated. It is essential that the titrations should be carried out slowly particularly near the end point, as the reaction between calcium or magnesium and EDTA is not very rapid. It is advisable to perform a blank titration on the acid, buffer and KCN.

If the volume of 0.002 M. EDTA used for the titration of the magnesium is x ml, that for the calcium y ml. that for the blanks b_1 and b_2 respectively and F is the molarity factor of the EDTA solution, the following formula will give the concentration of magnesium per 100 ml. serum or plasma.

$$\text{Mg } \therefore \text{ mg. per cent} = (x - b_1) \times .002 \times F \times 25.32 \times \tfrac{6}{5} \times 50$$
$$= (x - b_1) \times 2.92 \times F$$

The concentration of calcium per 100 ml. serum or plasma is given similarly by :

$$\text{Ca } \therefore \text{ mg. per cent} = (y - b_1) \times .002 \times F \times 40 \times 50$$
$$= (y - b_1) \times 4 \times F$$

Indetermining F, it is advisable to standardise the 0.002 M EDTA by following the technique for plasma with the standard solution.

RESULTS

Representative results shown in tables 1 and 2 were obtained from replicate estimations on various samples of plasma or serum. The column headed SD. shows the standard deviation of a single observation, column SE, the standard error of the mean and column C of V, the coefficient of variation or standard deviation as a percentage of the mean.

Table 1 shows the results obtained in the determination of the magnesium in a number of different samples of plasma. The standard deviation varies from .04 to .08 mg. per cent and the coefficient of variation from 1.2 per cent to 3.5 per cent with an average of 2.42 per cent. If the results from all the samples are pooled and an analysis of variance performed the variance within samples may be considered as the error variance of the method and the standard deviation of the method is .07 mg. per cent.

TABLE 1

THE DETERMINATION OF MAGNESIUM IN PLASMA OR SERUM

Sample No.	*Number of replicates*	*Mean conc. mg. %*	*S.D.*	*S.E.*	*C of V %*
9/C	5	2.66	.08	.04	3.0
9/H	6	2.52	.08	.03	3.2
2/C	5	2.00	.07	.03	3.5
2/H	5	2.94	.04	.02	1.4
11/C	5	2.58	.07	.03	2.75
13/C	4	2.00	.04	.02	2.0
X/C	6	3.52	.04	.02	1.2
Y/C	7	2.48	.05	.02	2.2
Z/C	17	5.36	.08	.02	1.5
Average					2.42

Table 2 shows the results obtained in the determination of the calcium in a similar series of samples. The standard deviation varied from .08 mg. per cent to .21 mg. per cent and the coefficient of variation from 0.7 per cent to 1.8 per cent. By analysis of variance the standard deviation is .16 mg. per cent.

TABLE 2

THE DETERMINATION OF CALCIUM IN SERUM OR PLASMA

Sample No.	*Number of replicates*	*Mean conc. mg. %*	*S.D.*	*S.E.*	*C of V %*
9/H	6	12.4	.21	.09	1.7
9/C	4	10.4	.19	.10	1.8
2/C	5	10.9	.08	.04	0.7
2/H	4	10.1	.08	.04	.8
13/C	6	10.0	.08	.03	.8
M/C	8	11.6	.19	.07	1.6
Average					1.23

The results in tables 3 and 4 were obtained by substituting 1 or 2 ml. of the standard solution for the distilled water in the calcium precipitation stage. The results are expressed as the amount of magnesium or calcium in 2 ml. plasma, that is the amount in each tube before precipitation.

Table 3 shows that for added magnesium the recovery ranges from 97 to 105 per cent with an average of 99.9 per cent while table 4 shows that for calcium the recovery ranges from 95 to 102 per cent with an average of 99.2 per cent. Both of these can be considered satisfactory.

TABLE 3

RECOVERY OF ADDED MAGNESIUM

Experiment No.	*No. of replicates*	*Mean plasma mg. mg. %*	*mg. Mg. added*	*Mg. found after addition*	*Percentage recovery*
1	3	.052	.02	.073	105
2	3	.038	.02	.058	100
3	6	.106	.04	.145	97.5
4	3	.048	.02	.068	100
5	6	.051	.02	.071	100
6	5	.053	.02	.0724	97
Average					99.9%

TABLE 4

RECOVERY OF ADDED CALCIUM

Experiment No.	*No. of replicates*	*Mean plasma Ca. mg.*	*mg. Ca. added*	*Ca found after addition*	*Percentage recovery*
1	4	.202	.100	.297	95
2	6	.233	.100	.335	102
3	4	.270	.200	.463	96.5
4	3	.216	.100	.315	99
5	4	.202	.100	.301	99
6	4	.209	.100	.310	101
7	6	.247	.100	.349	102
Average					99.2%

DISCUSSION

In the usual methods of determing calcium and magnesium as calcium oxalate and magnesium ammonium phosphate, the final stage involves reaction with the acid radical $(COO)_2^{=}$ or $PO_4^{\equiv}$; hence the precipitates must be well washed before titrating or developing a coloured complex. The washing process is very time consuming and there is always the possibility of loss. In the case of the determination of magnesium, the ratio of phosphate to magnesium in the precipitate can vary with the ambient temperature.

In the present method, the final stage is a direct titration of the calcium or magnesium with EDTA so the washing process is unnecessary and the determinations can be completed as soon as the initial precipitates are obtained. This is a distinct advantage in routine examination of samples. If calcium only is to be determined a suitable quantity of magnesium may be mixed with the buffer

and neutralised by EDTA before adding to the dissolved calcium precipitate.

The results presented show that the method is sufficiently reliable for clinical purposes as the determination of serum magnesium is, on the average, within 2.5 per cent while that for calcium is within 1.25 per cent.

CONCLUSIONS

A method for the determination of calcium and magnesium in serum or plasma is described. Calcium is precipitated as oxalate and magnesium as magnesium ammonium phosphate. The precipitated calcium and magnesium are titrated with 0.002 M. EDTA thus saving the necessity of washing the precipitates. The standard deviation of a single determination of magnesium was 0.07 mg. per cent and for Ca the standard deviation was 0.16 mg. per cent.

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THE VALUE OF THE COMPLEMENT-FIXATION TEST IN THE STUDY OF CANINE DISTEMPER COMPLEX AND RUBARTH'S DISEASE

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INTRODUCTION

Since 1949 series of experiments have been made on the application of the complement-fixation test (c.f.t.) to the study of the canine distemper-complex (DCX) and Rubarth's disease, also known as canine virus hepatitis (H.C.C.). In previous communications (Mansi, 1951, 1952, 1953; Hudson and Mansi, 1953) reference was made to this work on the complement-fixation test.

The c.f.t. has been applied widely during the last few years in the study of virus infections of man and animals. Various methods and techniques, including a number for the preparation of c.f. antigens and their purification have been reported, each being applicable to a particular virus infection, and while proving successful with one may not have been so in another. In this investigation it was necessary to prepare c.f. antigens and c.f. antisera specific for each virus infection. This was not an easy task owing to the number of infections to which dogs are susceptible. The intention throughout was to find a technique which would be practical, easy to perform and free from unnecessary complications.

Laidlaw and Dunkin (1931) were the first to apply the test to the study of canine distemper. In the present work, in addition to the classical strains of the canine distemper complex, other strains, for example, the distemperoid (Green, 1939), hard-pad (McIntyre, Trevan and Montgomerie, 1948), Glasgow S.123 (Mansi, 1951) and the egg-adapted strains of Haig (1948) and Cabasso and Cox (1949) have also been studied. Rubarth (1947) applied the test to the study of H.C.C. in dogs and found it useful in confirming clinical infections. Larin (1951a) attempted purification of H.C.C. virus-antigen by precipitating with methanol. Svenkerud (1952) in his studies on fox-encephalitis, drew attention to the importance of this test in the work of combating this condition.

In the present work, a comparative study of these antigen-antibody reactions (DCX and H.C.C.) is reported.

MATERIALS AND METHODS

Complement. Complement was provided by freshly-pooled guinea-pig sera, distributed in one or two millilitre amounts and treated in one of two ways : (1) frozen immediately at —20°C in which state the complement retained its activity for a period of three months; or (2) freeze-dried at once when complement activity was not reduced by a period of 2 years storage at 4°C or at room temperature (14 to 18°C).

The complement activity in haemolysing erythrocytes in the presence of specific haemolytic serum was determined by a simple titration of the complement (from 1/10 to 1/120) in ordinary physiological saline solution. The range of complement dilutions to be used in the test was determined by mixing positive and negative sera with positive and negative (normal) tissue-extracts which may be referred to as c.f. antigens, in the presence of different dilutions of the complement. This technique was essential because the titre of complement activity varied in the presence of different antigens and sera, when tested either alone or after mixing in the test proper.

Haemolytic system. (a) Three to five M.H.D. of haemolytic serum (anti-sheep erythrocytes), marketed by Messrs. Burroughs Wellcome, were used during this investigation.

(b) Sheep erythrocytes were obtained from sheep kept at the laboratory. The erythrocytes were washed 3 to 5 times with physiological saline solution. On many occasions the blood was kept in Alsever's solution for 5 to 6 weeks.

A 1.5 per cent suspension was finally prepared and to this an equal volume of diluted haemolytic serum was added.

Complement-fixing antigens. The two viruses under study are associated with the presence of the so-called "soluble antigens" in the infected animal's tissues. The soluble antigens are present in highest amounts in tissues rich in virus. The following table demonstrates the virus infection, the susceptible animal and the tissue of choice for preparation of provisional standard antigens.

TABLE 1

Virus infection	*Animal species*	*Tissue of choice*
Canine distemper complex	Ferret and Dog	Spleen and Lung
Rubarth's Disease	Dog and Fox	Liver

The method of preparation was that described by Casals and Palacios (1941). Final storage of the antigens was by freezing at –20° or freeze-drying, and differed from the original method described by them, which consisted of keeping the antigens in the ice box.

Details of the methods used were as follows :— Tissues were collected from experimentally infected animals which had been inoculated with the respective virus strain and then killed at, or shortly after, the height of the thermal and clinical reactions. They were tested for freedom from other viruses, by biological tests, and for freedom from contaminating bacteria. A 10 per cent suspension was prepared by grinding the tissue in a Ten Broeck grinder, using either normal physiological saline or 1 per cent pig serum-saline as diluent. After freezing at –20 to –70°C, the suspensions were thawed in a water-bath or incubator at 37°C, for 5 to 10 minutes, frozen and thawed again and the process repeated three to five times until the tissues were broken down, thus liberating all the virus-antigen. Finally the suspension was centrifuged at 3,000 r.p.m. for 15 minutes; the supernatant formed the complement-fixing antigen.

Complement Fixing Antisera

Rabbits. In the early stages of the work, sera were prepared from rabbits because of their resistance to natural and experimental infections. Before inoculation, rabbits were tested for the presence of c.f. and neutralising antibodies; none were found in the 74 rabbits used. Injection of virus suspensions were made once a week and, from the third week, the rabbits were bled weekly and the serum tested. When a good titre (1/16 to 1/32) was reached, the animal was inoculated with a further dose of virus and seven days later, at which time experience showed the presence of the highest titre of c.f. antibodies, the animal was bled. Immune animals were kept for long periods and, whenever serum was required, a further dose of virus was injected in order to stimulate antibody production; bleeding was then carried out seven days later.

Dogs. Sera were collected from vaccinated animals and from those which had recovered from experimental infections. Dogs were not injected until at least 2 samples of serum had been tested with negative results. After injection, 5 to 100 serum samples in all were collected at intervals of one week or more. In the later work, dogs were mainly used for DCX and H.C.C. c.f. antisera production. As with the rabbits, serum producing dogs were retained for subsequent bleeding.

Serum was separated from the blood within one hour of bleeding, especially in the case of samples from dogs; any delay in the separation usually resulted in a haemolysed serum. Sera were kept either frozen at −20°C or were freeze-dried at once. In the preparation of standard c.f. DCX anti-sera, the rabbits and dogs were immunized by the inoculation of supernatant obtained after centrifuging DCX infected ferret brains at 3000 r.p.m. for 30 mins. When used for the detection of c.f. antigen in ferret tissues, this procedure on rare occasions led to non-specific reactions. This was avoided by using dog sera stimulated by the inoculation of DCX infected dog's blood or avianized virus; but trouble from non-specific reactions has never been of great inconvenience. Dogs were stimulated with HCC infected dog's liver suspension for the production of standard c.f. HCC antibodies.

Inactivation of sera. Dog serum was inactivated at 64°C for 15 minutes, and rabbit serum for 20 minutes at 64°C. Inactivation was made immediately before the test was carried out. Unheated dog sera can be used in these antigen-antibody reactions, with very good results.

The Test Proper

Three preliminary tests were made before the test proper was carried out :— (a) titration of complement in the presence of constant amounts of antigens and antibodies, (b) titration of antigen in the presence of constant amounts of antibody and complement and (c) titration of antibody in the presence of constant amounts of antigen and complement.

From the results of the above tests, the complement dilution or dilutions were estimated. As controls, known positive and known negative sera were mixed with known infected and known normal tissue-extracts prepared in the manner described above.

Volume of reagents. Throughout all the tests constant amounts of reagents were added to each tube as follows :— 0.25 ml. serum dilution; 0.25 ml. tissue extract; 0.25 ml. complement dilution; 0.50 ml. haemolytic system (0.25 ml. haemolytic serum dilution and 0.25 ml. of 1.5 per cent washed sheep erythrocytes). The total volume was 1.25 ml. per tube.

A 1 cc "Agla" or tuberculin syringe with a 20 B.W.G./2"/ needle was used for distributing the amounts of each reagent. The barrel of the syringe was divided into 0.25 cc. sections with a bright colour to facilitate reading.

Time. After adding the serum, antigen and the complement to the tubes arranged in suitable racks, they were left on the bench for 15 minutes. They were then shaken well and incubated in a water bath or ordinary bacteriological incubator at 37°C for 60 minutes, after which 0.5 ml. of the haemolytic system was added to each tube. Forcible ejection of the sensitized cell suspension into the tubes from the syringe mixed the contents of the tubes efficiently and shaking was not necessary. When large numbers of tests were being made and many racks were used, automatic syringes were found convenient, but good care was necessary in washing them each time a new reagent was used. After adding the haemolytic system, a further incubation of 60 minutes at 37°C was required. The test was then read.

Readings. The following figures were adopted to represent the degree of fixation or of haemolysis.

4	=	complete fixation,	haemolysis	nil
3	=	75 per cent	,,	25 per cent
2	=	50 ,, ,,	,,	50 ,, ,,
1	=	25 ,, ,,	,,	75 ,, ,,
Tr.	=	traces of	,,	almost complete
0	=	no	,,	100 per cent

As these degrees of fixation or haemolysis were based on visual observation, two readings, at least (4 and 0), were unmistakable and thus the end point was based on either complete haemolysis in the case of titrating complement or complete fixation of complement in titrating and testing different sera and antigens. Other degrees of fixation or haemolysis were more difficult to assess, but were also taken into consideration.

RESULTS

Complement Activity

The freeze-dried complement was reconstituted, or the frozen complement was thawed and serial dilutions were made (1/10 to 1/20). These preliminary dilutions were necessary whenever a new batch of complement was prepared. Variable dilutions of complement were added to the antigen alone, to the serum alone and to

TABLE 2

THE EFFECT OF COMPLEMENT DILUTION ON THE TITRE OF AN ANTIGEN

Complement dilution	*H.C.C. positive serum* (1/4)							*Normal serum* (1/4)						
	Antigen dilution							*Antigen dilution*						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:10	1:20	1:40	1:80	1:160	1:320	1:640
1:10	4	2	tr.	0	0	0	0	0	0	0	0	0	0	0
1:20	4	4	4	2	0	0	0	0	0	0	0	0	0	0
1:30	4	4	4	4	4	0	0	0	0	0	0	0	0	0
1:40	4	4	4	4	4	2	0	0	0	0	0	0	0	0
1:50	4	4	4	4	4	4	tr.	0	0	0	0	0	0	0
1:60	4	4	4	4	4	4	3	0	0	0	0	0	0	0
1:70	4	4	4	4	4		4	0	0	0	0	0	0	0
1:80	4	4	4	4	4	4	4	4	2	tr.	0	0	0.	0
1:90	4	4	4	4	4	4	4	4	4	4	3	3	2	2

TABLE 3

Antigen dilution 1:20	*Antibody dilution* 1:4		*Complement dilutions* 1:10	1:20	1:20	1:40	1:50	1:60	1:70	1:80	1:90	1:100	1:110	1:120
Physiological saline	Physiological saline		0	0	0	0	0	tr	1	4	4	4	4	4
DCX ferret spleen	,,		0	0	0	0	0	0	0	0	0	2	2	4
Normal ferret spleen	,,		0	0	0	0	0	0	0	0	0	3	4	4
H.C.C. dog liver	,,		0	0	0	0	0	0	0	0	0	4	4	4
Normal dog liver	,,		0	0	0	0	0	0	0	0	1	3	4	4
Physiological saline	DCX. Dog Serum	*	0	0	0	0	0	0	0	0	0	3	4	4
DCX ferret spleen	,,		0	3	4	4	4	4	4	4	4	4	4	4
Normal ferret spleen	,,		0	0	0	0	0	0	0	0	tr	2	3	4
H.C.C. dog liver	,,		0	0	0	0	0	0	0	0	0	3	4	4
Normal dog liver	,,		0	0	0	0	0	0	0	0	0	2	3	4
Physiological saline	H.C.C. dog Serum		0	0	0	0	0	0	0	0	0	0	0	tr
DCX. ferret spleen	,,		0	0	0	0	0	0	0	0	0	0	3	4
Normal ferret spleen	,,		0	0	0	0	0	0	0	0	0	0	4	4
H.C.C. dog liver	,,	*	tr	4	4	4	4	4	4	4	4	4	4	4
Normal dog liver	,,		0	0	0	0	0	0	0	0	0	0		4
Physiological saline	Normal dog serum		0	0	0	0	0	0	0	0	0	0	2	4
DCX ferret spleen	,,		0	0	0	0	0	0	0	0	0	0	4	4
Normal ferret spleen	,,		0	0	0	0	0	0	0	0	0	0	4	4
H.C.C. dog liver	,,		0	0	0	0	0	0	0	0	0	tr	3	4
Normal dog liver	,,		0	0	0	0	0	0	0	0	tr	2	4	4

* specific antigen-antibody reaction

Notes on Table 3

In this table it will be observed that the minimum complement dilution necessary for complete haemolysis varies in the presence of physiological saline, antigen and serum. Antigens prepared, by the method described in this paper, were usually to some extent procomplementary.

Except for exceptional anticomplementary specimens, sera are usually procomplementary.

The use of too much complement prevents the recognition of a specific antigen-antibody reaction.

Normal antigen and serum of each animal species are included to permit detection of any non-specific (tissue) reaction.

The physiological saline sets of tubes are included to detect any possible anticomplementary offects of the sera or antigens used.

For a sensitive test one standard dose of complement cannot be employed. In testing an unknown serum or antigen a range of dilutions is used. When examining unknown serum the dose of this is fixed and complement is titrated in the presence of known positive and known negative antigens. When antigen is being examined, complement is titrated in the presence of known positive and negative sera. With this sample complement would be used at 1/40, 1/50, 1/60 & 1/70 as in table 4.

No common complement-fixing antigen is demonstrable between the two virus groups under study (DCX & HCC).

the serum and antigen combined. The plan of the method adopted is shown in table 3.

TABLE 4

Serum dilution 1:4 and reference	*Complement dilutions in the presence of*																	
	Distemper ferret spleen				*Normal ferret spleen*			*Canine hepatitis dog liver*					*Normal dog liver*					
	1:40	1:50	1:60	1:70	1:40	1:50	1:60	1:70	1:40	1:50	1:60	1:70	1:40	1:50	1:60	1:70		
Controls:																		
Physiological saline ..	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0		
DCX dog serum ..	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0		
H.C.C. dog serum ..	0	0	0	0	0	0	0	0	4	4	4	4	0	0	0	0		
Normal serum ..	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
1. Normal (negative) ..	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
2. DCX (weak) ..	0	0	3	4	0	0	0	0	0	0	0	0	0	0	0	0		
3. DCX (strong) ..	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0		
4. DCX (anti-complementary)	4	4	4	4	0	2	4	4	0	2	4	4	0	2	4	4		
5. H.C.C. (weak) ..	0	0	0	0	0	0	0	0	0	1	4	4	0	0	0	0		
6. H.C.C. (strong) ..	0	0	0	0	0	0	0	0	4	4	4	4	0	0	0	0		
7. DCX and H.C.C. (strong) ..	4	4	4	4	0	0	0	0	4	4	4	4	0	0	0	0		
8. DCX (non-specific with ferret spleen) ..	4	4	4	4	0	2	4	4	0	0	0	0	0	0	0	0		
9. Anti-complementary ..	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4		

Samples 1 to 9 indicate almost any possible reading which might be met.

It will be observed that with strong complement, fixation was only obtained with strong antigen. With weak antigen there was no fixation because of the presence of excess complement. With

TABLE 5

THE INTER-RELATIONSHIP BETWEEN THE DIFFERENT STRAINS OF CANINE DISTEMPER COMPLEX VIRUS

Animal Species	*Tissue*	*Strain reference*	*Complement dilutions in the presence of*											
			DCX. positive dog serum 1/4						*Normal dog serum* 1/4					
			1/20	1/30	1/40	1/50	1/60	1/70	1/20	1/30	1/40	1/50	1/60	1/70
Dog	Spleen	Glasgow S123 ..	0	tr	2	4	4	0	0	0	0	0	0	0
,,	,,	Normal ..	0	0	0	0	0	0	0	0	0	0	0	0
Ferret	,,	Laidlaw-Dunkin (W.D.V.)	3	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Wellcome Hard-pad (W.H.P.)	tr	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Green distemperoid ..	4	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Glasgow S. 123	4	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Normal	0	0	0	0	0	0	0	0	0	0	0	0
Chick-embryo	C.A.M.	Onderstepoort—adapted	0	0	0	0	0	0	0	0	0	0	0	0
,,	Embryo	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	Y-S	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	C.A.M.	Lederle—adapted ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	Embryo	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	Y-S	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	C.A.M.	Green—locally adapted ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	Embryo	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	Y-S	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	C.A.M.	Glasgow—locally adapted	0	0	0	0	0	0	0	0	0	0	0	0
,,	Y-S	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	C.A.M.	Normal												
,,	Embryo	,, ..	0	0	0	0	0	0	0	0	0	0	0	0
,,	Y-S	,,	0	0	0	0	0	0	0	0	0	0	0	0

1/20 etc. : complement dilution Serum dilution : 1/4 Antigen dilution 1/40 C.A.M. : chorio-allantoic membranes
Y-S : Yolk sac

weak complement, fixation occurred with weak antigen but there was also fixation with normal serum and it was clear that a

TABLE 6

THE INTER-RELATIONSHIP BETWEEN THE DIFFERENT STRAINS OF CANINE VIRUS HEPATITIS

Animal species	*Tissue*	*Strain reference*	*Complement dilutions in the presence of*													
			H.C.C. positive dog serum 1/4						*Normal dog serum* 1/4							
			1/20	1/30	1/40	1/50	1/60	1/70	1/20	1/30	1/40	1/50	1/60	1/70
Dog	Liver	Beckenham (Bec) ..	4	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Florent-Leunen ..	4	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Frömme	4	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Rubarth	4	4	4	4	4	4	0	0	0	0	0	0
Fox	,,	Beckenham (Bec) ..	4	4	4	4	4	4	0	0	0	0	0	0
,,	,,	Florent-Leunen ..	0	0	3	4	4	4	0	0	0	0	0	0
Chick-embryo	Yolk sac	Beckenham 1st passage ..	0	0	0	2	4	4	0	0	0	0	0	0
	Embryo	,, 3rd ,, ..	0	0	0	0	tr	4	0	0	0	0	0	0
		,, 10th ,, ..	0	0	0	0	0	0	0	0	0	0	0	0

1/20 etc. : complement dilutions Serum dilution 1/4 Antigen dilution 1/40

dilution of complement below 1/70 would be unsatisfactory. This sample of HCC antigen could be used in the routine examination of sera at a dilution of 1/40 to 1/60. The choice of an antigen dilution was not very critical when several dilutions of complement were used.

The effect of complement dilution on the titre of antibody. Titration on a c.f. antibody in the presence of its specific c.f. antigen was greatly affected by the use of variable dilutions of complement. An extract of a normal tissue similar to the tissue used as the positive antigen referred to for simplicity as "normal antigen" was included in the test to detect non-specific fixation if it should exist.

The results in tables 2, 3 and 4 show the necessity of choosing a complement dilution or dilutions to cover the range necessary to ensure detection of the minimum and maximum limits of specific reactions. Four or six complement dilutions were used in the presence of a suitable dilution of serum (1/4 to 1/8) and a suitable dilution of antigen (1/40 to 1/160). When the serum was the unknown, a 1:4 dilution was used and when the antigen was unknown, a dilution of antigen of 1:10 could be employed. If either the unknown serum or the unknown antigen gave no fixation in these dilutions it was very probable that they contained no complement-fixing antibody or antigen respectively.

From the titrations of complement activity illustrated in tables 2 and 3 it was found that the four following complement dilutions were suitable when this particular batch of complement was used; 1:40, 1:50, 1:60 and 1:70. Table 4 shows an example of a routine test.

Complement-fixing Antigens

In this section particular attention is paid to the relationship between the main virus groups under study.

The inter-relationship of the strains of the canine distemper complex. Tissues were collected from ferrets inoculated with the following strains of DCX:— Laidlaw-Dunkin ("W.D.V.") Wellcome hard-pad ("W.H.P."), Glasgow S 123 and Green distemperoid. The tissues were treated in a similar manner for antigen preparation. With all the strains, material collected from ferrets or dogs killed *in extremis* or which died from the infection, produced a soluble antigen common to all (Table 5). Variations in the end point occurred in some cases, but these did not indicate a qualitative difference; individual variations occur in ferrets and dogs inoculated with the same strain.

Avianised distemper virus-strains (Onderstepoort, Lederle, Green Glasgow S123, and Laidlaw-Dunkin) failed to yield c.f. antigen in chorio-allantoic membrane, yolk sac or embryo. Antigen was

demonstrated in material of the first two or three passages from original dog or ferret spleen, but not in materials of later passages even when they were shown to be infective or capable of immunising ferrets and dogs. If a virulent avianised virus which had lost its c.f. antigenicity was inoculated into a ferret it produced the clinical disease. After the ferret died or was killed *in extremis* the c.f. antigen

TABLE 7

PROPERTIES OF "HCC" AND "DCX" COMPLEMENT-FIXING ANTIGENS

Antigen and treatment	*Antigen dilution in the presence of*													
	H.C.C. positive serum ¼							*Normal serum* ¼						
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/10	1/20	1/40	1/80	1/160	1/320	1/640
H.C.C.														
(1) F.T.C. fresh	4	4	4	4	4	4	0	0	0	0	0	0	0	0
(2) Butanol														
a. colloid	4	4	4	4	4	0	0	0	0	0	0	0	0	0
b. deposit	4	4	0	0	0	0	0	0	0	0	0	0	0	0
c. colloid after F.T.C. 5 times	4	4	4	tr	0	0	0	0	0	0	0	0	0	0
d. deposit after F.T. 5 times	4	0	0	0	0	0	0	0	0	0	0	0	0	0
(3) Methanol														
a. supernatant	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b. precipitate	4	4	2	1	0	0	0	0	0	0	0	0	0	0
(4) Heat														
a. 4°C for 1 wk.	4	4	4	4	4	0	0	0	0	0	0	0	0	0
b. Room T° 19°C for 1 wk.	4	4	4	4	3	0	0	4	4	4	4	0	0	0
c. 37°C for 1 wk.	4	4	4	3	0	0	0	4	4	0	0	0	0	0
d. 64°C 120 min.	4	4	4	0	0	0	0	0	0	0	0	0	0	0
e. 100°C 5 min.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(5) Freeze-drying	4	4	4	4	4	2	0	0	0	0	0	0	0	0
D.C.X.														
(1) F.T.C. fresh	4	4	4	4	4	4	2	0	0	0	0	0	0	0
(2) Butanol														
a. colloid	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b. deposit	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(3) Methanol														
a. supernatant	0	0	0	0	0	0	0	0	0	0	0	0	0	0
b. precipitate	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(4) Heat														
a. 4°C for 1 wk.	4	4	4	4	4	4	0	4	2	0	0	0	0	0
b. Room T° 19°C for 1 wk.	4	4	4	4	2	0	0	4	4	2	0	0	0	0
c. 37°C for 1 wk.	4	4	2	0	0	0	0	4	4	4	0	0	0	0
d. 64°C 5 mins.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
e. 100°C 5 mins.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(5) Freeze-drying	4	4	4	4	4	4	0	0	0	0	0	0	0	0

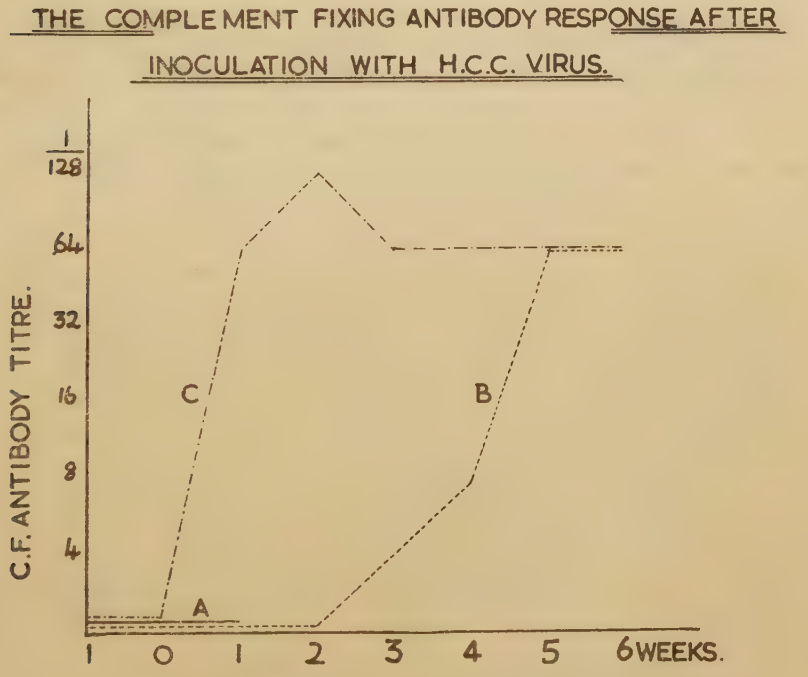
F.T.C. : Frozen, thawed and centrifuged

was demonstrable in its tissues. The distribution of distemper antigen in the various tissues of dogs and ferrets suffering from distemper is given in table 8.

The inter-relationship of the strains of the canine hepatitis virus group. Various strains of the virus of Rubarth's disease and fox encephalitis (Florent-Leunen, Rubarth, Fromme and Beckenham) were used. The Beckenham strain has been referred to in previous work as "Bec", (Mansi, 1953). Materials collected either from foxes which showed fox encephalitis or from dogs showing canine virus hepatitis, after inoculation with the above strains were examined. These samples, when extracted, yielded a common complement-fixing antigen (Table 6). The distribution of such antigen in the different tissues is shown in table 8.

Virus propagated on the chorio-allantoic membrane or in the yolk sac of infected chick-embryos, failed to give such a soluble antigen after a few egg-to-egg passages.

Figure 1.



A————. No complement fixing antibodies. Animal died with the infection at the end of the first week.

B– – – – – –. Complement fixing antibodies which gradually appeared from the end of the third week and onward, reach their highest titre by the 5th week. Animal showed clinical symptoms of the infection and recovered.

C —. —. —. —. An immune animal which had lost its complement fixing antibodies. On reinoculation with the homologous virus, complement fixing antibodies appeared within the first week, accompanied by no sign of clinical infection.

Properties of the c.f. antigens. The "DCX" virus antigen is heat-labile, destroyed by heating at 56°C for five minutes and no antigen can be prepared by methanol or butanol treatment (Table 7).

The "HCC" virus antigen is heat-stable, resists heat at 64°C for 2 hours but is destroyed by boiling for 5 minutes. The antigen can be precipitated by methanol (Larin, 1950) or extracted by butanol, the latter method being superior to the former. Both preparations are less rich in antigen than those obtained by the simple method of freezing, thawing and centrifuging (Table 7).

Complement-Fixing Antibodies

Complement-fixing antibodies of the two viruses under investigation are demonstrable in susceptible animals by the end of the third or fourth week after inoculation with the respective viruses. H.C.C. complement-fixing antibodies may remain in the circulation for only 12 weeks but in some cases have persisted for 2 to 3 years. DCX complement-fixing antibodies remain for a shorter period, usually between three to ten weeks and, very rarely, for as long as fifteen weeks. However, an animal immune to either of these virus infections from which complement-fixing antibodies have disappear-

TABLE

DISTRIBUTION OF SOLUBLE ANTIGEN IN DIFFERENT TI

Virus		*Canine distemper complex*									
Animal Condition		*Dog*						*Ferret*			
		Infected		*Immunised*			*Infected*		*Immunised*		
Route of infection		I/V	I/N & S/C	S/C	S/C	S/C	I/P	I/C	I/P	I/P	I/P
Fate of animal		K+	K+	K	K	K	K+	K	K	K	K
Interval between infection and collection of tissues (in days)		4	12	41	120	214	5	10	31	67	97
Tissues examined	Liver	1/80	1/40	0	0	0	1/320	1/640	0	0	0
	Lung	1/80	1/320	0	0	0	1/640	1/640	0..	0	0
	Spleen	1/80	1/640	0	0	0	1/640	1/640	1/20	0	0
	Kidney	1/20	1/80	0	0	0	1/80	1/80	1/40	1/40	1/20
	Urinary bladder	1/80	1/80	0	0	0	1/80	1/40	0	0	0
	Mesenteric lymph node	1/160	1/320	0	0	0	1/640	1/640	0	0	0
	Tonsil	1/80	1/80	0	0	0	1/40	1/80	0	0	0

I/V : intravenous I/N : intranasal S/C : subcutaneous I/P : intraperitoneal

I/C : intracerebral I/C : intraocular K : killed in clinically normal condition

ed, will regain them on reinoculation with the homologous virus; a relatively high titre of antibody will be detectable by the 7th day and may remain for some weeks (Fig. 1). A dual infection will result in the production of both complement-fixing antibodies (Table 9).

DISCUSSION

The dose of complement must be selected after the titration of complement is made in the presence of constant dilutions of known positive antigens and their specific c.f. antibodies, known normal tissue extracts and normal sera corresponding to the animal species involved in the test (Table 3). Thus when titration of antigen or antiserum is required one selects as the dose of complement the minimum amount necessary to give complete fixation in a specific antigen-antibody reaction and a negative reading in its absence. In ordinary routine tests a series of four to six complement dilutions, very carefully chosen, added to constant dilution of sera and constant dilution of antigens has repeatedly given satisfactory results (Table 4).

The virus-infections under study are associated with the presence of complement-fixing soluble antigens specific to each virus and some

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SSUES IN DISTEMPER AND RUBARTH'S DISEASE

	Rubarth's Disease (Canine Virus Hepatitis)											
	Dog										*Fox*	
	Infected				*Immunised*						*Infected*	*Recovered*
I/P	I/O	I/P	I/V	Contact	Instillation in the Eye						I/O	I/O
K	+	K+	+	+	K	K	K	K	K	K		
120	3	5	7	8	6	16	21	41	120	214	4	18
0	1/320	1/320	1/320	1/640	1/20	1/10	0	0	0	0	1/320	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	1/80	1/160	1/10	1/20	1/10	0	0	0	0	0	1/320	0
0	1/40	1/40	1/160	0	1/80	1/10	0	0	0	0	1/160	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	1/160	1/160	1/160	1/160	1/10	1/10	0	0	0	0	1/80	0
0	1/40	1/40	1/80	1/40	1/320	1/10	0	0	0	0	1/84	0

K+ : killed in extremis + : dead 1/80 : titre of antigen

This table indicates typical representatives of each clinical condition.

of the properties of these antigens have been examined (Table 7). Strains of the DCX virus group (Laidlaw-Dunkin, (W.D.V.); Green distemperoid; Hard pad, (W.H.P.); Glasgow, S123 and other locally isolated strains) all share a common soluble antigen. This antigenic identity has been mentioned in previous publications (Mansi, 1951, 1952) and has been confirmed by other biological and immunological studies (Mansi, 1951, 1952; Dedie, 1951; Fankhauser, 1951; Cabasso, 1952). Thus hard-pad disease, instead of being considered as a separate disease caused by a virus distinct from that of canine distemper (MacIntyre, Trevan and Montgomerie, 1948), is in fact one of the strains of the canine distemper complex virus group. Different strains of this virus may produce, on first isolation, symptoms in dogs and ferrets different from those caused by the classical Laidlaw-Dunkin strain. Some workers advise the elimination of the term "hard-pad" to avoid clinical confusion (Fankhauser, 1951).

Strains of Rubarth's disease isolated from dogs and those of fox encephalitis isolated from foxes also share one common antigen. (Table 6). This antigen is serologically quite distinct from that of the distemper complex group and if a given tissue shows fixation with specific sera for both or if an unknown serum reacts with both standard antigens, a dual infection is proved. There is no common antigen shared by the two virus-groups.

TABLE

Period	*Weeks*	*Complement-fixing antibody*									
		DCX. virus									
		DCX antigen				*H.C.C. antigen*				*DCX*	
		Complement dilutions									
		1/40	1/50	1/60	1/70	1/40	1/50	1/60	1/70	1/40	1/50
Before inoculation	3	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	0
After inoculation	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	1	0	0	0	0	0	0
	3	2	4	4	4	0	0	0	0	0	0
	4	4	4	4	4	0	0	0	0	0	0
	5	tr.	1	4	4	0	0	0	0	0	0
	6	0	0	3	4	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0

DCX virus : Glasgow S123 virus

Sera tested here are not anticomplementary

The distribution of virus-antigens of the DCX and of H.C.C. in the body of infected animals has been investigated (Table 8). Animals inoculated with HCC. virus behave differently according to the method of infection adopted. A dog inoculated intraperitoneally, intravenously or intraocularly with a fatal result, shows a high titre of antigen in the liver, while a dog infected by the instillation method (Mansi, 1953) shows a low titre in the liver and a higher titre in the tonsil and thyroid. A dog killed at the height of the reaction (6th day) will show more antigen than a dog killed at the end of the reaction (19th day), while recovered animals killed after 12 days or more will usually show no c.f. antigen in their tissues. A very small proportion still have a low titre in the kidney.

In spite of the fact that the virus produces fox encephalitis, very little c.f. antigen is present in the central nervous system and the pulmonary system has not shown any indication of the presence of this specific soluble antigen. A dog infected with DCX virus and killed at the height of the clinical reaction will show the presence of the antigen in many tissues but not to the same extent or concentration as in the ferret.

It is of considerable importance to collect serum samples before inoculation or before exposure to infection and again the following week. The results of the c.f. test together with the clinical history in the inoculated animal enable a proper appraisal of its immunological state, i.e. whether it is susceptible or immune (Fig. 1).

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response in dogs inoculated with

HCC. virus						*DCX.A + H.C.C. viruses*							
antigen		*HCC antigen*				*DCX antigen*				*H.C.C. antigen*			
		Complement dilutions				*Complement dilutions*							
1/60	1/70	1/40	1/50	1/60	1/70	1/40	1/50	1/60	1/70	1/40	1/50	1/60	1/70
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	3	4	0	0	0	2	0	0	0	2
0	0	4	4	4	4	4	4	4	4	4	4	4	4
0	0	4	4	4	4	tr	2	4	4	4	4	4	4
0	0	4	4	4	4	0	tr	2	4	4	4	4	4
0	0	4	4	4	4	0	0	tr	3	4	4	4	4
0	0	4	4	4	4	0	0	0	1	4	4	4	4
0	0	4	4	4	4	0	0	0	1	4	4	4	4
0	0	4	4	4	4	0	0	0	tr	4	4	4	4
0	0	4	4	4	4	0	0	0	0	4	4	4	4
0	0	4	4	4	4	0	0	0	0	0	tr	3	4

DCX.A: Avianized, inoculated subcutaneously

HCC virus : liver emulsion instilled in the conjunctival sac of the dog

In order that the two c.f. antibodies shall be distinct and specific for their respective virus groups, a great deal of care must be taken

TABLE 10

Serum dilution 1:4	*Antigens dilution* 1:100 Complement dilutions	*DCX positive ferret spleen*				*Normal ferret spleen*				*HCC positive dog liver*				*Normal dog liver*				*Physiological saline*			
		20	30	40	50	20	30	40	50	20	30	40	50	20	30	40	50	20	30	40	50
DCX positive serum	Unheated	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Heated 56°C 30 mins.	4	4	4	4	0	0	1	4	0	2	4	4	0	0	0	2	0	0	0	1
	Heated 60°C 20 mins.	4	4	4	4	0	0	tr	4	0	2	4	4	0	0	0	tr	0	0	0	1
	Heated 64°C 15 mins.	0	4	4	4	0	0	0	tr	0	0	0	1	0	0	0	tr	0	0	0	tr
HCC positive serum	Unheated	0	0	0	0	0	0	0	0	4	4	4	4	0	0	0	0	0	0	0	0
	Heated 56°C 30 mins.	0	0	tr	3	0	0	0	3	4	4	4	4	0	0	0	1	0	0	0	tr
	Heated 60°C 20 mins.	0	0	tr	3	0	0	tr	3	4	4	4	4	0	0	0	tr	0	0	0	0
	Heated 64°C 15 mins.	0	0	0	0	0	0	0	0	4	4	4	4	0	0	0	0	0	0	0	0
Normal serum	Unheated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Heated 56°C 30 mins.	0	tr	1	2	0	0	tr	2	0	0	tr	2	0	0	0	tr	0	0	0	0
	Heated 60°C 20 mins.	tr	2	4	4	0	tr	4	4	0	3	4	4	0	0	0	tr	0	0	0	0
	Heated 64°C 15 mins.	0	0	0	2	0	0	0	3	0	0	0	tr	0	0	0	0	0	0	0	0

The effect of inactivation of dog serum on the specificity of the antigen-antibody reaction

in the preparation of the standard sera. The following are some of the difficulties which can be encountered.

Non-specific reactions can be avoided by using as far as possible, a homologous tissue extract for immunisation. Where this is not possible and a heterologous tissue-extract is used, a tissue other than the one used as antigen, should be chosen. For example, if antigen has been prepared from the spleen, brain may be used as inoculum to immunise and stimulate dogs and rabbits for the production of anti-distemper serum. However, with the sera of a very small number of immunised animals a non-specific reaction has been encountered, especially when ferret brain has been used as antigen. When such non-specific reactions occur it is advisable to avoid using the serum from such animals when testing ferret tissues. This serum is, however, suitable for testing dog material. The use of uninactivated dog sera has reduced this difficulty (Table 10).

The presence of more than one complement-fixing antibody in the serum of an animal is not unusual. Usually it is a disadvantage to use such a serum to detect complement-fixing antigen in an infected tissue. The time factor may, however, be of help if an H.C.C. antiserum is required since an animal infected with H.C.C. may show a titre of antibody for 2 to 3 years, while it has been unusual for canine distemper antibody to persist for more than 15 weeks. An animal, which is immune to either of the viruses under study, at a stage when complement-fixing antibodies have ceased to be demonstrable, can be stimulated to produce a mono-specific c.f. antibody by reinoculation with the virus required.

Anti-complementary properties have been rare in sera and in antigens prepared by freezing material (serum or tissue) or freeze-drying. If they have occurred, additional freezing, thawing and centrifuging, or the addition of normal sera has often removed them. Uninactivated dog serum gives a better reaction as demonstrated in table 10. (This finding followed an observation made by Gambles in relation to the contagious bovine pleuro-pneumonia antigen-antibody reaction).

The complement-fixation test has been most valuable in identifying these virus infections. In the early stages of the clinical disease the presence of H.C.C. antigen may be detectable in the serum (Larin, 1951b). In the case of an animal dying from either H.C.C. or DCX infection, extracts from tissues, preferably liver, spleen and mesenteric lymph node prepared in the manner described above, will indicate the presence or absence of the specific soluble c.f. antigens. A serum sample collected from a dog at the time of infection and another collected a week or two later will definitely confirm the virus infection. Thus an unknown serum, and an unknown antigen can be tested in the presence of a known standard antigen or a known standard serum respectively. The technique adopted

makes it possible to obtain a result within three hours from the time of receiving the material. Compared with biological, immunological and pathological tests the c.f. test has proved useful and convenient.

CONCLUSIONS

The paper describes in detail the technique of the complement-fixation test used to study the viruses of the canine distemper complex and Rubarth's disease. The necessity for using more than one dilution of complement is explained.

There is no antigenic relationship between the two groups of virus-strains. On the other hand it is not possible to distinguish the different strains of either group by this test.

The distribution of soluble antigen in the bodies of infected, recovered and immune animals has been studied as has the antibody response in animals inoculated with these viruses.

The results obtained with the complement-fixation test have confirmed observations on these viruses obtained by biological, immunological and pathological methods.

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THE CHEMOTHERAPY OF EXPERIMENTAL FOWL CHOLERA IN FOWLS (*GALLUS DOMESTICUS*)

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INTRODUCTION

The more active sulphonamides have been shown by a number of investigators to be of value in the prevention and treatment of the experimental and natural forms of fowl cholera in several species of domestic birds (Saurat, 1944 ; Delaplane and Higgins, 1948 ; De Volt, 1948 ; Hilbert and Kiser, 1948 ; Kiser, Prier, Bottorff and Green, 1948 ; Peterson, 1948 ; Alberts, 1950 ; Mikes, 1951 ; Mukarovsky, 1951). Favourable results have also been obtained with penicillin (Queen and Quortrup, 1946 ; Zuydam, 1952) and streptomycin (McNeil and Hinshaw, 1948).

The present paper records investigations on the use of sulphamezathine, sulphamerazine, furazolidone, penicillin, dihydrostreptomycin, chloramphenicol, aureomycin, terramycin and quinoxaline–1 : –4 D_1–N–oxide in the treatment of the experimental form of the disease in fowls.

MATERIALS AND METHODS

Chickens. White Leghorn chickens from a pedigree flock that had been closed for five years were employed when they were either one-week-old (approximate weight 60 g.) or nine-weeks-old (approximate weight 0.75 kg.). The former are referred to throughout as chicks and the latter as chickens. The chicks had not been sexed but the chickens were all males. They were kept on wire-mesh floors and were fed on a standard proprietary mash while under experiment.

Pasteurella septica *cultures.* Six strains of *Pasteurella septica,* 2479, 3407, 3195, 8141, 8282, 8283, obtained from the National Collection of Type Cultures, Colindale, London, were used in the *in-vitro* sensitivity tests of which 2479 and 3407 were of avian origin. Unless otherwise stated, all infection experiments were carried out with 2479.

Chemotherapeutic agents. Penicillin was employed either as crystalline sodium penicillin G of a potency of 1617 units per mg. or as procaine penicillin suspended in a mixture of sodium carboxymethylcellulose and a sorbitan mono-oleate polyoxalkylene derivative (" Distaquaine "). Dihydrostreptomycin was used as the sulphate and aureomycin and terramycin as the hydrochloride, buffered with sodium glycinate. Chloramphenicol was employed in powder form for oral administration and in a soluble form (Allen & Hanbury) for injection. Sulphamezathine and sulphamerazine were used as sodium salts for sensitivity tests, parenteral injection and for adding to drinking water. Furazolidone was employed in powder form and quinoxaline 1–: –4–D_1–N–oxide in aqueous solution. All agents given by mouth were dispensed in gelatin

capsules. Some were also given mixed in the mash and in the drinking water. All injections were made intramuscularly. The choice of route of administration and dosage was decided mainly after considering the results of *in-vitro* sensitivity tests and of observations on blood levels of these agents in chickens (Smith, 1954).

In-vitro *sensitivity tests.* A suitable range of two-fold serial dilutions of each chemotherapeutic agent was made in 5 ml. of liquid medium. Each series of tubes and a control tube containing medium but no agent was then inoculated with a suitably diluted culture of *P. septica.* The tubes were incubated for 24 hours at 37°C and read, the lowest concentration of the agent that completely inhibited bacterial multiplication, as judged by visual examination, being recorded as the minimal inhibitory concentration. The medium used for the sulphonamide tests was nutrient broth from which sulphonamide-inhibitors had been removed according to the method of Evans (1948). The inoculum was approximately 100 bacteria. For tests with the other agents, ordinary nutrient broth at pH 7.2 and an inoculum of approximately 100,000 bacteria was used.

Method of producing infection. Chickens were infected by the intramuscular injection of 0.1 ml. of a 24 hour broth culture of *P. septica* containing approximately 100 million bacteria per ml. For chicks, the culture was first diluted 100 times before being injected in the same volume of medium. These doses produced a 100 per cent. infection rate in both chicks and chickens. After infection, observations were made on chicks for at least 7 days and on chickens for 14 days ; preliminary observations showed that deaths very rarely took place after these periods of time.

RESULTS

The in-vitro *sensitivity of* P. septica *to chemotherapeutic agents.* The sensitivity of six strains of *P. septica* to eight chemotherapeutic agents is shown in Table 1. Penicillin was the most active agent tested, 0.1 to 0.2 μg./ml. being sufficient to inhibit the growth of all six

TABLE 1

THE *IN-VITRO* SENSITIVITY OF SIX STRAINS OF *P. SEPTICA* TO CHEMOTHERAPEUTIC AGENTS

Agent	*Minimal inhibitory concentration (μg./ml.) for strain number*					
	2479	*3407*	*3195*	*8141*	*8282*	*8283*
Penicillin	0.1	0.2	0.2	0.2	0.2	0.1
Chloramphenicol	0.2	0.8	0.8	1.6	0.2	0.2
Terramycin	0.4	0.8	0.8	0.8	0.4	0.4
Dihydrostreptomycin	0.8	0.8	0.8	0.4	0.8	0.8
Aureomycin	0.4	1.6	3.2	3.2	1.6	0.8
Sulphamerazine	0.4	1.6	—	0.8	1.6	1.6
Sulphamezathine	0.8	1.6	—	1.6	1.6	1.6
Furazolidone	2.0	2.0	4.0	1.0	1.0	1.0

strains. These strains were also quite sensitive to chloramphenicol (0.2 to 1.6 μg./ml.) terramycin (0.4 to 0.8), dihydrostreptomycin (0.4 to 0.8), aureomycin (0.4 to 3.2), sulphamerazine (0.4 to 1.6) and sulphamezathine (0.8 to 1.6). They were only moderately sensitive to furazolidone (1.0 to 4.0). Quinoxaline-1 : -4-D_1-N oxide was only tested against strains 2447 and 3407 ; both were inhibited by a concentration of 10 μg./ml.

The Treatment of Infected Chicks

The results of treating groups of 30 infected chicks with different agents twice daily for four days at a dose level of 25 mg./kg. is shown in Table 2. The most satisfactory method was terramycin intramuscularly followed, in order of comparative value, by the same

TABLE 2

THE EFFECT OF ADMINISTERING CHEMOTHERAPEUTIC AGENTS, 25 MG./KG., TWICE DAILY FOR FOUR DAYS TO INFECTED CHICKS

		No. of 30 chicks that died on the following days after infection						*No.*	*%*
		1	*2*	*3*	*4*	*5*	*6*		
Terramycin	intramuscular	0	0	0	0	0	0	0	0
Terramycin	oral	0	1	0	1	0	0	2	6.7
Aureomycin	oral	2	0	0	0	0	1	3	10.0
Chloramphenicol	oral	0	1	2	2	0	0	5	16.7
Dihydrostreptomycin	intramuscular	1	5	2	0	0	0	8	26.7
Penicillin	intramuscular	3	2	3	0	0	0	8	26.7
Quinoxaline-oxide	intramuscular	1	4	2	2	1	0	10	33.3
Sod. sulphamerazine (100)	intramuscular	3	8	2	0	3	3	19	63.3
Sulphamerazine (100)	oral	10	6	2	3	1	1	23	76.6
Sulphamerazine	oral	13	8	3	0	1	1	26	86.7
Sulphamezathine	oral	11	9	4	0	2	2	28	93.3
Furazolidone	oral	21	3	4	0	0	0	28	93.3
None	—	27	3	0	0	0	0	30	100.0

(100) = chemotherapeutic agent was used at 100 mg./kg. instead of the usual level o 25 mg./kg.

The first three doses of each agent were given at 6, 12 and 24 hr. after infection ; the remainder were given every 12 hours.

agent and aureomycin, both given orally, chloramphenicol dihydrostreptomycin, penicillin and quinoxaline-oxide intramuscularly. The intramuscular administration of aureomycin was not studied owing to its irritant nature. Poor results were obtained with sulphamerazine and sulphamezathine even when they were used at four times the usual dose level. Furazolidone was valueless.

The results of repeating the previous experiment using terramycin at different dose levels, intramuscularly and orally, are shown in Table 3. This shows that 25 mg./kg. was more satisfactory than 5 and 1 mg./kg. and that the intramuscular route was superior to the oral route.

TABLE 3

THE EFFECT OF ADMINISTERING DIFFERENT LEVELS OF TERRAMYCIN, INTRAMUSCULARLY AND ORALLY, TWICE DAILY FOR FOUR DAYS TO GROUPS OF 30 INFECTED CHICKS

Dose level (mg./kg.)	*Route*	*Total died* No.	%
25	intramuscular	0	0
	oral	2	6.7
5	intramuscular	5	16.7
	oral	18	60.0
1	intramuscular	11	36.6
	oral	25	83.3
0	—	29	96.7

The effect of two doses of each chemotherapeutic agent, both of 25 mg./kg., given at 6 and 24 hours after infection to groups of 30 chicks is illustrated in Table 4. Two new forms of treatment were

TABLE 4

THE EFFECT OF ADMINISTERING CHEMOTHERAPEUTIC AGENTS, 25 MG./KG., TO CHICKS AT 6 AND 24 HOURS AFTER INFECTION

Agent	*Route*	*No. of 30 chicks that died on the following days after infection*						*Total died*	
		1	*2*	*3*	*4*	*5*	*6*	No.	%
Terramycin (in liquid paraffin)	intramuscular	4	0	0	0	0	0	4	13.3
Terramycin	intramuscular	1	4	0	0	0	0	5	16.7
Chloramphenicol	intramuscular	0	6	1	1	0	0	8	26.7
Terramycin	oral	8	0	4	0	0	0	12	40.0
Dihydrostreptomycin	intramuscular	2	5	6	0	1	0	14	46.7
Penicillin	intramuscular	2	8	6	2	0	0	18	60.0
Procaine penicillin	intramuscular	1	6	8	2	1	0	18	60.0
Quinoxaline-oxide	intramuscular	5	4	10	1	1	1	22	73.3
Aureomycin	oral	8	7	5	5	1	0	26	86.7
Chloramphenicol	oral	7	17	1	0	2	0	27	90.0
None		27	2	0	0	0	0	29	96.7

included, terramycin in liquid paraffin and procaine penicillin. The dose of procaine penicillin used in this and succeeding experiments was 40,000 units/kg., approximately 40,000 units being contained in 25 mg. of the ordinary form of penicillin that was being tested. The sulphonamides were not studied in view of the comparatively poor results obtained previously. Best results were obtained with terramycin in liquid paraffin and in its usual aqueous vehicle, both given by the intramuscular route. Good results were also obtained with

chloramphenicol administered intramuscularly. These were much better than when this agent was given orally. Intramuscular chloramphenicol was followed in order of comparative value by oral terramycin, dihydrostreptomycin, procaine and aqueous penicillin and quinoxaline-oxide. Oral aureomycin was of little value.

The results of single doses of six chemotherapeutic agents, 25 mg./kg., given 6 hours after infection to groups of 30 chicks, are shown in Table 5. Terramycin intramuscularly, in water or liquid paraffin,

TABLE 5

THE EFFECT OF ADMINISTERING A SINGLE DOSE OF CHEMOTHERAPEUTIC AGENT, 25 MG./KG., TO CHICKS AT 6 HOURS AFTER INJECTION

Agent	*Route*	*No. of 30 chicks that died on the following days after infection*						*Total died*	
		1	*2*	*3*	*4*	*5*	*6*	*No.*	*%*
Terramycin	intramuscular	3	2	1	0	0	0	6	20.0
Terramycin (in liquid paraffin)	intramuscular	5	1	0	0	0	0	6	20.0
Dihydrostreptomycin	intramuscular	2	16	3	0	0	0	21	70.0
Quinoxaline-oxide	intramuscular	9	4	7	2	1	1	24	80.0
Chloramphenicol	intramuscular	2	22	0	0	0	0	24	80.0
Procaine pencillin	intramuscular	11	15	1	1	0	0	28	93.3
None		28	2	0	0	0	0	30	0

was greatly superior to the other agents. Somewhat similar results were obtained with single doses of terramycin given intramuscularly when strain 3407 was used as the infecting strain instead of the customary strain 2479 ; three of 30 chicks died. In another experiment in which the single dose of terramycin given 6 hours after infection was equivalent to either 25, 10 or 5 mg./kg. the numbers of 30 chicks that died were 3, 6 and 16 respectively. When a single dose of terramycin, 25 mg./kg. was given at 12 instead of 6 hr. 21 of 30 chicks died ; one died before treatment commenced.

TABLE 6

THE EFFECT OF THE CONTINUOUS ADMINISTRATION OF SULPHAMERAZINE, SULPHAMEZATHINE AND FURAZOLIDONE TO CHICKS BEFORE AND AFTER INFECTION

Agent	*Method of Administration*	*No. of 30 that died on the following days after infection*						*Total died*	
		1	*2*	*3*	*4*	*5*	*6*	*No.*	*%*
Sulphamerazine	0.2% in mesh	1	1	1	0	0	0	3	10
Sulphamerazine	0.1% in drinking water	0	0	0	0	0	0	0	0
Sulphamezathine	0.2% in mash	0	1	0	0	0	0	1	3.3
Sulphamezathine	0.1% in drinking water	0	0	0	0	0	0	0	0
Furazolidone	0.04% in mash	30	0	0	0	0	0	30	100
None		30	0	0	0	0	0	30	100

Administration of the chemotherapeutic agents was commenced 2 days before infection and continued for 6 days.

The effect of the continuous administration of sulphamerazine, sulphamezathine and furazolidone to chicks before and after infection. The results of the continuous administration of sulphamerazine and sulphamezathine, 0.2 per cent in mash or 0.1 per cent in the drinking water, to groups of 30 chicks for 6 days commencing 2 days before infection are shown in Table 6. The results for 0.04 per cent furazolidone in the mash are also included. The sulphonamides were highly effective in this form of preventive treatment ; furazolidone was valueless.

The Treatment of Infected Chickens

The results of administering single doses, 25 mg./kg.. of terramycin, dihydrostreptomycin and chloramphenicol intramuscularly to groups of 20 chickens at 6 hours after infection are illustrated in Table 7. Only one chicken treated with terramycin, compared with 9 in the dihydrostreptomycin group, 12 in the chloramphenicol group and 18 in the untreated control group, died. All the survivors appeared normal at the end of the observation period of 14 days.

TABLE 7

THE EFFECT OF ADMINISTERING SINGLE DOSES OF TERRAMYCIN, DIHYDROSTREPTOMYCIN AND CHLORAMPHENICOL, 25 MG./KG., INTRAMUSCULARLY, TO CHICKENS AT 6 HOURS AFTER INFECTION

Agent	*No. of 20 chickens that died on the following days after infection*										*Total died*	
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*No.*	*%*
Terramycin	0	0	0	0	0	0	0	0	1	0	1	5
Dihydrostreptomycin	0	0	1	1	0	2	0	2	1	2	9	45
Chloramphenicol	1	2	4	0	0	1	1	1	1	1	12	60
None	2	8	2	2	2	1	0	1	0	0	18	90

The results of delaying the treatment of groups of 20 chickens with single doses of terramycin or dihydrostreptomycin, 25 mg./kg. intramuscularly, until 24 hours after infection are illustrated in Table 8. Of the chickens selected for this experiment, 15.4 per cent died within 24 hours ; the figures quoted in Table 8 refer to groups of 20 chickens alive when treatment was commenced. Once again, the superiority of terramycin was demonstrated. Apart from longer survival times there was little difference between the dihydrostreptomycin group and the untreated controls. Three of the terramycin group and one of the dihydrostreptomycin group were slightly lame 14 days after infection due to joint involvement ; the remainder appeared perfectly well.

TABLE 8

THE EFFECT OF ADMINISTERING SINGLE DOSES OF TERRAMYCIN AND DIHYDROSTREPTOMYCIN, 25 MG./KG., INTRAMUSCULARLY, TO CHICKENS AT 24 HOURS AFTER INFECTION

Agent	*No. of 20 chickens that died on the following days after infection*									*died*	
	2	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*No.*	*%*
Terramycin	1	0	0	0	0	0	0	0	0	1	5
Dihydrostreptomycin	5	1	1	2	3	1	1	0	0	14	70
None	9	1	4	1	0	0	0	0	0	15	75

Chickens that died before treatment was commenced were not included in the groups of 20

DISCUSSION

In multiple dosage several of the agents were quite satisfactory in the treatment of the experimental infection in chicks and chickens, but the superiority of intramuscular terramycin was shown when only one or two doses were employed. This is particularly important in relation to the economics of poultry management. Apart from the fact that the strains of *P. septica* were very sensitive to terramycin *in-vitro,* an important reason for the success of terramycin in the experimental form of the disease is its slow rate of excretion by poultry (Smith, 1954). The superiority of the intramuscular over the oral route was not unexpected as terramycin is poorly absorbed from the alimentary tract of chickens (Smith, 1954). These results indicate that intramuscular terramycin might be of value in the treatment of natural fowl cholera in poultry. It would also seem worthy of trial in *P. septica* infection of other domestic animals and man.

Although comparatively poor results were obtained with the sulphonamides when administration was commenced after infection, the excellent results obtained when they were given continuously in the food or drinking water commencing before infection confirms the observations of others on their value in prophylactic flock treatment.

CONCLUSIONS

Terramycin given intramuscularly was superior to penicillin, procaine penicillin, dihydrostreptomycin, chloramphenicol, aureomycin and quinoxaline–1 : 4–D_1–N–oxide in the treatment of experimental fowl cholera in chicks and chickens. Poorer results were obtained with sulphamerazine and sulphamezathine than with all these agents. Furazolidone was of no value.

One intramuscular injection of terramycin, 25 mg./kg., was usually sufficient to cure the disease in chicks and chickens infected intramuscularly 6 and 24 hours previously.

The continuous administration of suphamerazine and sulphamezathine, 0.2 per cent in the mash or 0.1 per cent in the drinking water was very effective in controlling the disease when it was commenced before infection.

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STUDIES ON *Trichostrongylus axei*

IV Factors in the causation of pathogenic Effects by *T. axei*

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INTRODUCTION

The pathogenesis of *T. axei* in sheep, kept both on a low plane of nutrition and on a full diet, has been recorded in two papers by Gibson (1954a, 1954b). Since relatively few sheep were used in these experiments it was thought profitable to repeat the work for confirmation of the results.

Accordingly an experiment was carried out for this purpose but it was also arranged to check the effect of the infestation on the appetite of the experimental sheep. The results of this experiment suggested a further investigation to ascertain whether factors other than the effect on appetite were involved in the causation of disease by *T. axei*. These two investigations are recorded in this paper. All reference to the part of the first experiment designed for confirmation of previous work has been omitted but complete details of methods and results are available from this laboratory should any reader wish to have them.

Effect of *T. axei* on the Appetite of Infected Sheep

Materials and Methods

Eight pairs of Dorset Horn twin lambs free from trichostrongylid infestation were used and were divided into four groups A, B, C and D in such a manner that of four pairs of twins one of each twin pair fell in group A and the other in group B, with a similar distribution between groups C and D.

The two groups of lambs A and B were kept on a high plane of nutrition, their diet consisting of hay and concentrates mixture fed *ad lib*. The concentrates mixture contained bran, oats, flaked maize and linseed cake. Groups C and D were kept on a diet of hay only fed to appetite. Throughout the course of the experiment the weekly food consumption of each group was calculated by subtraction of the amount of food left at the end of each week from the amount allocated to the group at the beginning, care being taken that food rejected by the animals was not discarded but retained and weighed along with the surplus food at the end of each week.

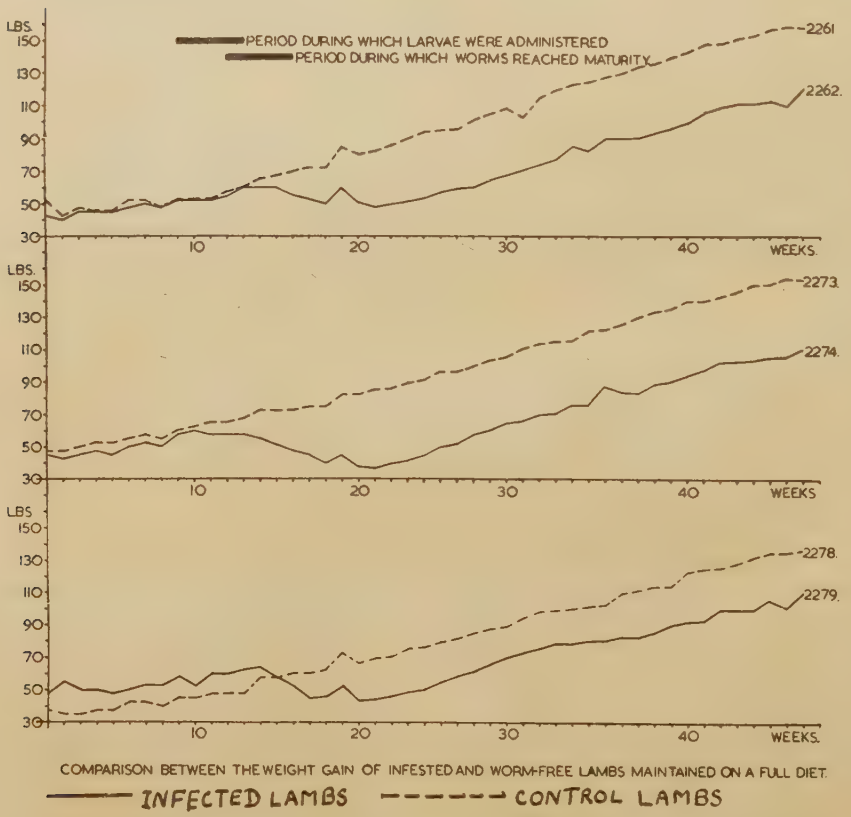
At the beginning of the experiment the lambs were approximately four months old and the administration of infective larvae of *T. axei* was begun in groups A and C two months later, each lamb receiving a daily dose of 4,000 infective larvae until a total of 100,000 had been given to each.

All the animals were weighed on Thursday mornings, food and water having been removed overnight so as to avoid weight changes resulting from the ingestion of large quantities of food and water just prior to weighing.

Results

The results have been summarised by means of four figures. Fig. 1 shows the weight curves of three of the lambs from group A together with those of their twins from group B. Data on food consumption is recorded in Figs, 2, 3 and 4, the results have been expressed as average weight of food consumed weekly per sheep, and the upper diagram of each figure gives the results for the infected group whilst the lower one refers to the corresponding control group.

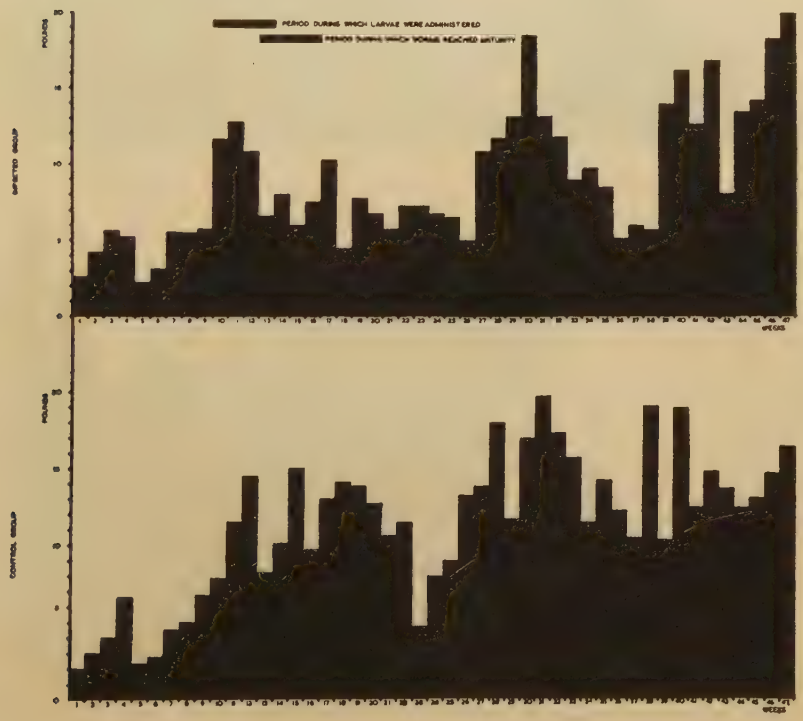
Fig. 1



A study of Fig. 1 shows the effect of worm burden on the weight gain of infested sheep. It will be observed that throughout the experiment the three worm-free control sheep (2261, 2273 and 2278) gained weight at a steady rate. Their twins (2262, 2274 and 2279) began by gaining weight in a similar fashion, but between the

twelfth and twenty-first weeks all three sheep lost weight. Egg count data, not recorded in this paper, shows that the beginning of this period coincides with the time when mature worms were first present in the sheep and ends with the time when developing resistance enabled the sheep to throw off all but a very small part of their infestation. After the twenty-second week, however, these three infected lambs gained weight steadily and it is remarkable that their rate of weight gain was the same as that of the control sheep, but they remained, on the average some 35 pounds lighter than these latter. It is very clear, therefore, that an attack of parasitic gastro-enteritis can cause serious loss in a flock of sheep, even though successful treatment of the disease results finally in the sheep becoming fat and apparently well grown animals. Superficial observation of a flock of affected animals gives the impression that after recovery from an outbreak of parasitic gastro-entritis, weight gain is rapid and that the sheep are little the worse off for the experience. The results given above suggest that this impression is erroneous, for under the experimental conditions described in this paper the effect of the disease was never overcome and it is probable that the same effect occurs in a natural outbreak.

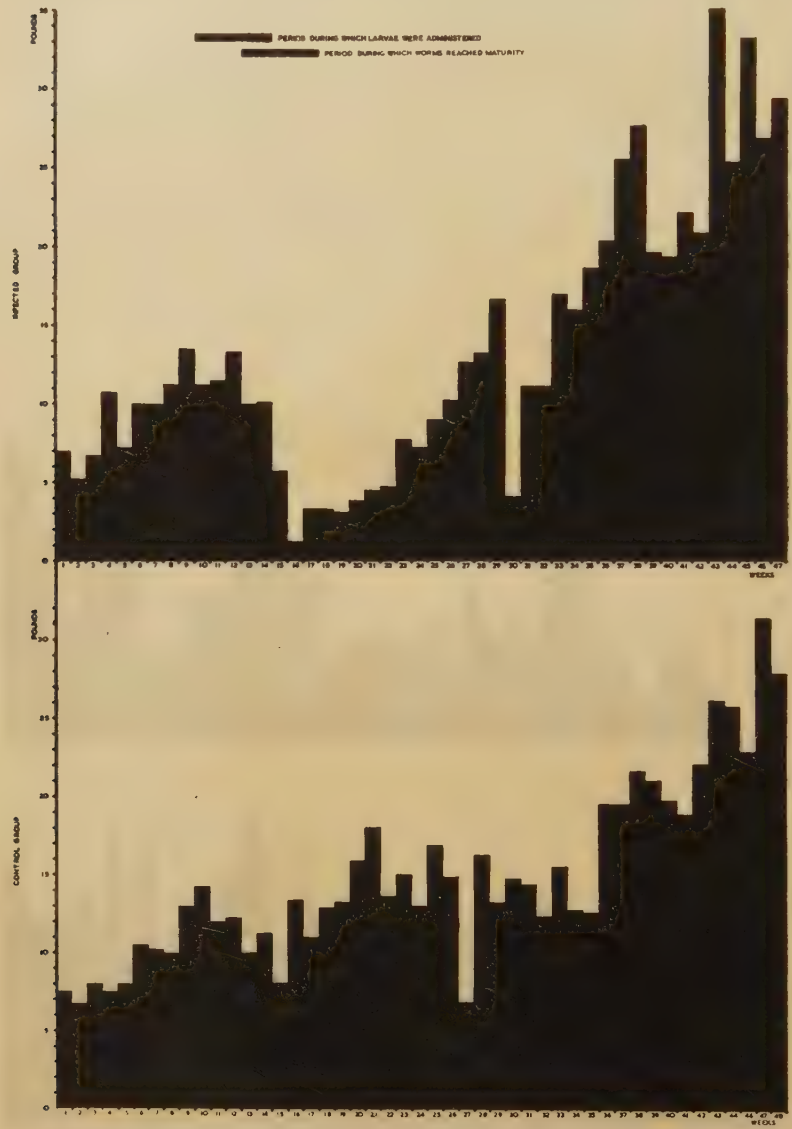
Fig. 2



AVERAGE WEEKLY CONSUMPTION OF HAY BY THE TWO GROUPS OF SHEEP RECEIVING A FULL DIET

The observations on food consumption are also very interesting. Fig. 2 shows the hay consumption of the sheep receiving a full diet and it will be observed that from the twelfth to the twenty-fifth week hay consumption of the infested sheep was considerably less than that of the control sheep but before the twelfth week and after the twenty-first hay consumption of the two groups was comparable. From Fig. 3 it will be observed that the effect of infestation on the consumption

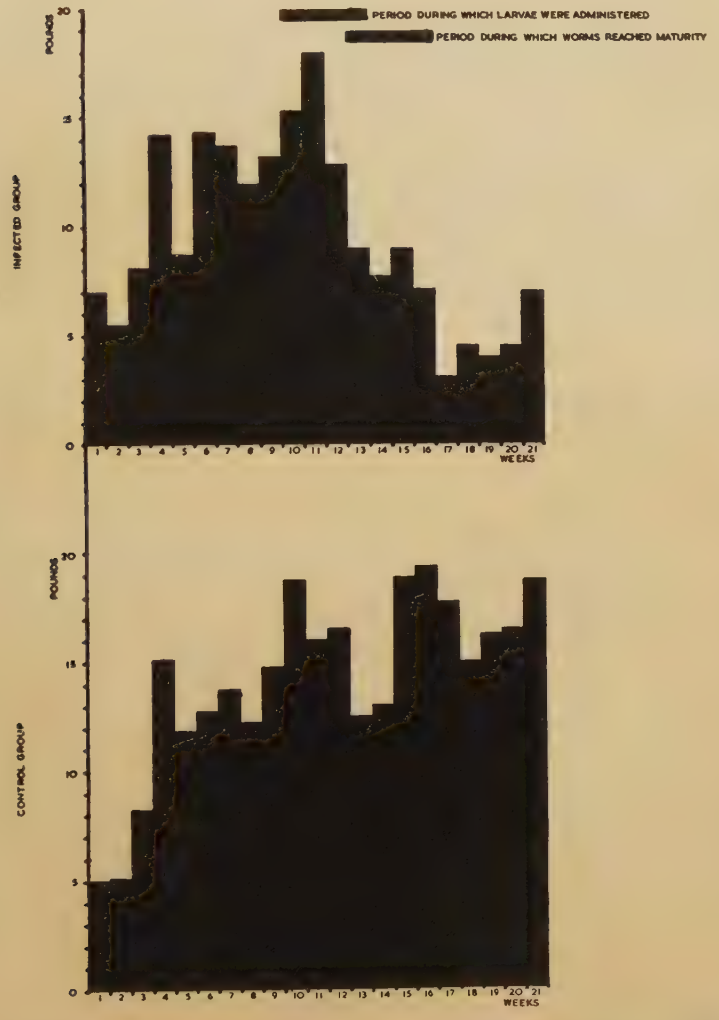
Fig. 3



AVERAGE WEEKLY CONSUMPTION OF CONCENTRATES BY THE TWO GROUPS OF SHEEP RECEIVING A FULL DIET

of concentrates was even more marked. At the twelfth week a sharp decline in the amount of concentrates consumed by the infested sheep took place and this depression of appetite persisted until the twenty-second week, after which appetite was rapidly regained until at the end of the experiment the infested sheep were consuming food at much the same rate as the controls. The control group showed no such depression of appetite ; the amount of concentrates consumed by this group increased quite regularly week by week.

Fig. 4



AVERAGE WEEKLY CONSUMPTION OF HAY BY THE TWO GROUPS OF SHEEP RECEIVING A POOR DIET

Fig. 4 shows the effects seen in the group of sheep receiving a diet of hay only. It is seen that after the worms reached maturity the appetite of infested sheep decreased and this tendency continued but observations were terminated by death of the sheep. In contrast to this the control sheep, which received hay only, increased their hay consumption throughout the period of observation.

Effects of *T. axei* Other than Depression of Appetite, on Infested Sheep

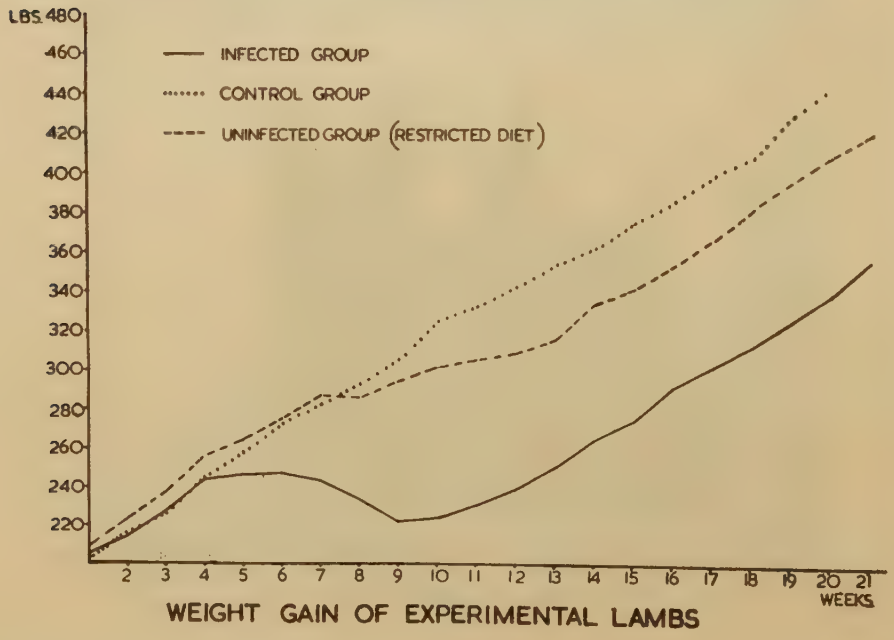
Having shown that depression of appetite resulted in sheep infested with *T. axei,* a further experiment was carried out to ascertain whether this was the sole effect of the worms on their host.

Three groups of three trichostrongylid-free sheep were used in this investigation and one group was infected with *T. axei* in the manner described above. The other two groups were kept uninfected. The infected group and one of the uninfected groups were fed hay and concentrates *ad lib,* the food consumption being ascertained by weighing as in the experiment described above. The second uninfected group was restricted in the amount of food it was allowed to consume during any one week to that eaten by the infected group during the previous week. Weight gain of all three groups of sheep was recorded weekly.

Results

The total weight gain for the three groups of sheep is recorded in Fig. 5. In drawing this diagram, allowance has been made for the

Fig. 5



fact that the group receiving a restricted diet were one week behind the other two groups. It will be seen that the weight gain of the worm-free sheep on a restricted diet was intermediate between that of the other two groups. Clearly some factor other than depression of appetite is involved in the causation of disease by *T. axei*, for if this were the only factor operating, the weights curves of the infected group and the restricted diet group should be identical.

DISCUSSION

The results of the first experiment show clearly that depression of appetite is an important factor in the causation of disease by *T. axei*. Depression of appetite by nematode infestations has been described by other authors. Andrews, (1939), using sheep infested with *T. vitrinus*, *T. colubriformis* and a few *T. axei*, noted depression of appetite in affected lambs but the same author, in 1938, when working with lambs affected with *Cooperia curticei* found that both infested and control lambs ate approximately the same quantity of food although the infested lambs showed a decrease in ability to convert food into gain in weight. Laurence, Groenewald, Quin, Clark, Ortlepp and Bosman (1951) found that appetite for hay was depressed in sheep carrying mixed infestation of *Haemonchus contortus* and *Oesophagostomum columbianum* but that appetite for concentrates was unaffected. It is interesting that those species which have been shown to depress appetite cause severe disease with gross damage in the abomasum or small intestine. Andrews (1939) remarks that the affected sheep appeared depressed, suffered from abdominal pain and were unable to eat all the feed given to them. *Haemonchus contortus*, used in the experiments by Laurence *et al*, also causes lesions in the abomasum which are doubtless accompanied by pain, and there is no doubt that the severe abomasitis found in the acute stage of infestation with *T. axei* (Gibson, 1954c) is accompanied by considerable pain which makes the animals disinclined to eat. Since appetite is so dramatically curtailed during the acute stage of infestations with *T. axei* it is not surprising that rapid loss of weight occurs during that period.

Although the present work shows that factors additional to depression of appetite are involved in its pathogenesis, no work was carried out to ascertain what these factors might be. Spedding (1954) has, however, demonstrated that in addition to depression of appetite, *T. axei* causes decrease of digestive efficiency in lambs. This is undoubtedly part, at least, of the additional effect revealed in the second experiment described in this paper.

CONCLUSIONS

An experiment carried out on four groups of four lambs, two of which received a full diet and two a poor one, confirmed the results recorded in two previous papers concerning the pathogenesis of

T. axei infestation in sheep. This section of the experiment has not been recorded in detail in the present paper.

In addition, the present experiment demonstrated clearly that infestations of *T. axei* cause a marked reduction in appetite during the acute stage of the infection. The resulting loss in weight was as much as 35 pounds per lamb, and it was found that the animals did not make good this loss after throwing off their infestation but always remained lighter than the controls.

A further experiment involving a group of three infested lambs and two groups of three worm-free lambs, in which one of these latter groups was restricted in its food intake to the amount consumed by the infested lambs, showed a factor in addition to depression of appetite to be operating in the causation of pathogenic effects by *T. axei*.

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CANINE DISTEMPER VIRUS IN THE FERRET

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INTRODUCTION

Final proof of the association of a specific virus with a disease usually requires recovery or isolation of the infective agent during the acute phase of the disease or at autopsy. The choice of the test host, whether it be a laboratory animal, an embryonated hen's egg, or a tissue culture, will be dictated by convenience. The test host should, however, be free from active or latent infection, and it must also be highly susceptible to the virus, preferably more so than the natural host. The choice of test hosts for canine distemper virus is not large, and the ferret, introduced by Laidlaw and Dunkin (1926), still remains the conventional test host for the isolation of the virus and for the propagation of its strains.

Since Laidlaw and Dunkin (1926) described the behaviour of distemper virus in the ferret it has generally been accepted that the ferret, when infected with the virus, regularly suffers from an acute disease which is characterised by a remarkably constant pathogenesis. Because of this evidence, and the cross-immunity tests using strains of the above virus, it was accepted that distemper virus was a single entity, and that the response of the ferret to infection was comparable in all essentials with that of the dog (Laidlaw and Dunkin, 1926).

The research which followed has, however, gradually shown that the disease, canine distemper, did not quite fit the description given by Laidlaw and Dunkin. In connection with this, as well as with failures in producing immunity by accepted methods of active immunisation, antigenic variations or mutations of distemper virus have been suggested, but these have not been experimentally proved. The extensive controversial literature on this subject has recently been reviewed by Whitney and Whitney (1953) and need not be repeated here. Two investigations, however, will be mentioned because they showed strikingly contradictory results which may now be looked at in the light of the experimental data reported in this paper.

The first of these is by MacIntyre, Trevan, and Montgomerie (1948) who introduced the name " Hard Pad " for a virus which differed immunologically from the " Laidlaw and Dunkin " strain of distemper virus. Although recovery after either " Laidlaw and Dunkin " or " Hard Pad " virus infections left the dog immune to homologous virus only, the authors suggested that " Hard Pad " virus was in some way related to the virus described by Laidlaw and Dunkin.

The second is by Mansi (1952) who conducted a comparative study of " Hard Pad " and " Laidlaw and Dunkin " strains obtained from MacIntyre and his co-authors. Mansi found these strains to be indistinguishable in the cross-immunity and complement fixation tests, although the course of infection with " Hard Pad " virus differed in the ferret. No suggestions were made, by Mansi, as to the reasons for the

contradictory behaviour of " Hard Pad " virus in the two independent laboratories ; neither has it been reported whether the original strains of " Hard Pad " virus remain unchanged after many passages.

The determination that a virus isolated from an infected animal is the agent of canine distemper ordinarily suffices to establish the diagnosis. Since the virus strains may vary considerably it is desirable that the diagnosis of virus distemper be extended beyond the identification of the virus described by Laidlaw and Dunkin so as to embrace adequate characterisation of the properties of the various strains of the distemper virus complex. The present work records an investigation of the behaviour in the ferret of 55 virus strains isolated from as many cases of " clinical distemper " in various parts of Britain during the years 1952-54.

MATERIALS AND METHODS

Ferrets. Only healthy adult ferrets from litters in which no disease was observed and no deaths had occurred were used. The ferrets were fed on the same diet before and throughout all the experiments. When under experiment they were kept in specially designed galvanized bins, with lids and all conventional precautions were taken to preclude any possibility of cross infection.

Temperatures. These were taken by the same two experienced animal technicians throughout all the experiments. Although the temperature of ferrets may sometimes rise due to excitement it can be avoided if a little patience and skill is used in handling these animals. Temperatures of ferrets were taken at the same time once daily.

Virus inoculum. The standard volume of virus inoculum for each ferret was 1 ml. in the form of, either, a 10 per cent tissue suspension in buffered glucosol, whole blood, or cerebrospinal fluid. Specimens for preparing virus inocula were taken from dogs in the acute phase of the disease, or at an autopsy conducted within twelve hours after death. During the preparation of the inoculum the virus was kept on ice. All inoculations were made intraperitoneally.

Strains of virus were usually passaged without storage, but when the virus was stored for a short period this was done in sealed containers at −40° C. or −76° C. As a rule the inocula were sterile and no filtration was required ; contaminated inocula were filtered through a Kieselguhr candle.

RESULTS

The behaviour of the 55 strains on their isolation and passages in the ferret was so strikingly different as to justify their classification into 3 groups, A (8 strains), B (17 strains), C (30 strains).

Group A

A typical response is recorded in Fig. 1 which shows the reaction of 9 ferrets, 8 of which were inoculated with virus material taken from dogs with " clinical distemper," and one with the laboratory

strain of virus (" Laidlaw and Dunkin "). Fig. 2 is designed to illustrate the typical pattern of the biological behaviour of A Group strains on their successive passages in the ferret.

The data recorded in Figs. 1 and 2 present a number of points of interest. In the first place, it will be seen that strains of the A Group regularly produced an acute febrile illness in all inoculated ferrets. The incubation period was remarkably constant, varying from 3 to 4 days ; on only a very few occasions has it been as long as 6 days in other experiments with A group strains. The first sign of the disease was a form of remittent fever with exacerbations and remissions, but without intermissions ; in some ferrets the fever was of a marked diphasic character. During the high fever lasting from 5 to 13 days, the prodromal period depending upon the duration of the illness, the animal appeared normal. After the prodromal period, anorexia or conjunctivitis marked the onset of specific signs. There was no regularity in the development of the specific signs produced with the various strains of this group. Anorexia of short duration appeared in all ferrets, while conjunctivitis and vesicles around the mouth appeared in many, though not all, and diarrhoea only in a few. Congestion of the skin of the feet and of the lower part of the abdomen appeared very irregularly and no nervous manifestations were observed.

Following the appearance of the specific signs the illness progressed rapidly, the animal becoming weaker and the coat very rough. Death usually occurred between the second and the fifth day after the specific signs appeared.

Considered as a whole Figs. 1 and 2 show that, although the behaviour of the A group strains varied slightly, they possessed a similar virulence and pathogenicity for the ferret. They also produced an acute illness of the same pattern after as many as 15 consecutive passages (Fig. 2). Group A virus was usually present in the blood during the febrile episodes or after death, and, thus, could be isolated from practically any tissue. In brief the main signs of infection with A group strains (Figs. 1 and 2) were high fever, with the appearance of anorexia towards the end of the illness. Conjunctivitis and vesicles around the mouth appeared with less regularity and death usually occurred within 2 weeks of infection. The strains did not change their pathogenicity for the ferret during as many as 15 consecutive passages and produced a disease of the same pattern in all inoculated ferrets.

Group B

It was a little puzzling that, whereas the 8 strains of group A had a degree of virulence for ferrets which was remarkably constant in the sense that no inoculated ferrets survived longer than 20 days, most of the remaining strains given in the same dose and by the same route displayed great variation in their virulence from passage to passage. Several series of experiments were conducted in order to

investigate the response of the ferret to these virus strains. Results typical of the 17 strains classified as group B are represented by two strains. (Figs. 3 and 4).

Fig. 3 shows the response of ferrets to the Sheffield virus strain. Ferret F.61 was inoculated with the whole blood taken from a dog with " clinical distemper," but this inoculation produced neither an obvious illness nor febrile reaction sufficient to suggest that virus was present in the blood.

After the death of the dog the virus was isolated in ferret F.62 which was inoculated with a spleen and lung tissue suspension. It can be seen from Fig. 3 that in ferret F.62 the virus produced a similar illness to that produced with the A group strains (Figs. 1 and 2), except that the anorexia was of longer duration.

This strain was passaged from ferret F.62 to ferrets F.75, F.76, and F.77 (Fig. 3) as well as to ferret F.61 (inoculated previously with the whole blood of the dog). The results show clearly that if the dog's blood contained virus it conferred upon ferret F.61 no immunity to the challenge inoculation, as this ferret developed an illness, similar to that in ferret F.62, and died. The fresh ferrets (F. 75, F.76, and F.77) responded differently to the virus inoculum. One ferret (F.75) developed relapsing fever with anorexia, lasting for 8 days, as well as conjunctivitis, and succumbed on the 23rd day. Of the other 2 ferrets, one (F. 76) did not show a significant febrile reaction while the other (F.77) developed relapsing fever ; both these ferrets, however, had occasional anorexia for 1 or 2 days but no other signs of illness. One month after the first inoculation both ferrets were challenged with A type virus (F.74). One of the 2 challenged ferrets (F.77) developed an acute illness of a pattern rather similar to that produced with the A type virus (Fig. 2) and died on the 11th day. The other challenged ferret (F.76) survived for several months and was eventually destroyed.

The essential features of the above results were obtained with the other strains of this group which during subsequent passages produced either more, or less, virulent variants. A typical example of this change in virulence is recorded in Fig. 4 which shows that the Ely strain increased in virulence after the 1st passage and resembled the group A strains. On the 2nd passage through 2 simultaneously inoculated ferrets the strain produced 2 variants, one of high and one of low virulence. On the 3rd passage ferret F.119 was inoculated with the variant of high virulence (F.103). As this ferret displayed an illness which was similar to that in ferret F.103 it was hoped that the A type virus had at last been recovered, but the 4th passage (F.122 and F.123) again showed two variants, and on the 5th passage the only ferret inoculated (F.124) did not die although the inoculum originated from the variant which resembled A group virus (F.123). More ferrets inoculated with the virus from F.123 gave similar results to the previous passages. There was no difference in the behaviour of B group strains whether the standard inoculum was used or dilu-

tions ranging up to 10^{-6}. These results suggest that the 17 strains of distemper virus described as group B during their passages through ferrets were changing in two directions to either high or low virulence for the ferret. The highly virulent variants resembled A group virus, while the variants of low virulence induced in the ferret an illness of quite a different type with an incubation period which was sometimes as long as ten days. Inoculated ferrets developed relapsing fever. Mouth lesions and conjunctivitis were inconstant. Anorexia frequently occurred throughout the illness, and in fatal cases was of long duration. Hardening of the pads and nervous manifestations were not observed with any one of the 17 strains. Mortality was about 70 per cent of the ferrets inoculated.

Group C

A typical example of the pattern of behaviour in the ferret of the 30 virus strains in this group is shown in Fig. 5 (F.110 and F.10). It is obvious that the illness induced in ferret F.110 with a Lincoln strain, and in ferret F.10 with a Lancaster strain, differs considerably from that induced in ferret F.2 with the A type, Rutland strain.

Of the several interesting points emerging from the analysis of Fig. 5 the one with immediate bearing on subsequent experiments is the finding that the ferret is not a highly susceptible host for the successful propagation of C type virus. The other feature of the C type virus was that the illness induced in ferrets by the virus before (Fig. 6), and during (Fig. 7), passaging differed fundamentally from the illness produced by A type strains. After an incubation period, which was sometimes as long as 10 days, the C type produced in the ferret a relapsing fever with moderate elevations of temperature and often with subnormal temperatures during remissions. Mouth lesions, conjunctivitis, and diarrhoea were very inconstant, and it seemed that the most important sign was anorexia which was of long duration in some ferrets and probably contributed to the fatal outcome. The duration of illness was never less than 3 weeks and was often as long as 2 or 3 months. Photophobia without conjunctivitis, was common in ferrets inoculated with C type virus, while in ferrets inoculated with the A type virus photophobia was either not observed, or, if present, it always appeared simultaneously with severe conjunctivitis. It may be noted from Fig. 4 that photophobia without conjunctivitis was sometimes caused by variants of the B Group strains. In the course of many experiments with the 30 strains of C type in ferrets no hardening of the pads was observed. Nervous manifestations such as paralysis, tremors, etc., were very occasionally seen and were not reproduced on subsequent passages.

With regard to the change during successive passages, the C type strains behaved similarly to the B type. As can be seen from Fig. 7 (Lancaster strain) the graphs suggest a similarity to the B type, except the duration of anorexia which was longer in infection with the

Lancaster strain than with any strain in group B. In later experiments, however, this strain failed to produce highly virulent variants during passages, and the great majority of variants of the other strains of C group were of low virulence. The mortality of ferrets inoculated with virus of C type was not above 50 per cent. It, appears, therefore, that the dominant direction of the change in the C group strains is towards low virulence for the ferret.

When a mixture of A and C type strains was inoculated into a ferret the more vigorous A type always overgrew the C type, and in all subsequent passages only the A type virus developed with complete loss of the C type strain. This phenomenon occurred with all the strains used.

As is the case of B group strains, some ferrets which did not die after inoculation with C group strains had no immunity to the A group and died after being challenged, while others developed febrile reactions but recovered. Examples of A, B, and C group strains were isolated from different dogs affected with distemper in a single epizootic.

It will need much further work before the nature of the process by which the change in distemper virus complex can be determined and antigenic differences of emerging variants revealed. Work is concontinuing in an attempt to find data which might assist in differentiating between viruses causing "clinical distemper," and, in elucidating the mechanism of immunity.

DISCUSSION

To a large extent the results reported in this paper are concerned with providing a background for subsequent studies on the structure of the distemper virus, and on the immune response in the ferret and dog. It is not intended that the behaviour in the ferret of the 55 virus strains so far studied should be the final classification of virus types, nor is it suggested that a particular type of virus is associated with a particular form of illness in the dog, a task probably beyond present knowledge. With these reservations the data presented in this paper can be discussed under two main headings.

The first is the striking difference in the biological behaviour of virus strains in group A as compared with group C. The disease produced in the ferret with C group strains did not in the least resemble that produced by distemper virus in the ferret as described by Laidlaw and Dunkin (1926).

The second point is concerned with the phenomenon of variability observed in the majority of the distemper virus strains studies (47 out of 55). In order to simplify the demonstration of this phenomenon, as shown by experiments on ferrets, the 55 strains studied were grouped under A, B, and C. A type strains did not change on successive passages ; B type strains produced variants resembling either A or C type ; C type strains produced variants

resembling B, but never A type strains. These experiments also showed that the ferret was not a very susceptible host for the successful propagation of C type strains. The phenomenon of variability can be explained by the existence of two virus types, A and A_1, within the group ; the former did not noticeably change their biological behaviour in the ferret on successive passages, while the latter produced variants of C type. Some C type strains produced A_1, variants on successive passages, while the others (C_1) produced variants less virulent for the ferret than the paternal strains.

There are probably only two alternatives to account for the findings reported in this paper. The first is that the above data have no significance since the differences observed in the behaviour of the virus might be due either to the varying susceptibility of the ferrets to the virus used for experiments, or to the variations in the proportion of live and dead virus particles and antibody in the inocula used. The second potential argument against the validity of the phenomenon of variability and the existence of virus types is that various types of virus have been isolated from different dogs affected in a single epizootic, and that the meaning of the word "type" is acceptable only for an immunological type. Since variations in virulence are more difficult to measure than immunological characters, virulence is not an acceptable criterion for virus grouping. It is also felt that it might be a legitimate criticism that B and C type viruses were not in fact " true " distemper viruses.

There is, however, much evidence against the above arguments. Using the methods employed in this investigation it was impossible to check every virus variant of B, or C groups on its immunological relationship to the A group strains. It is evident, however, from Figs. 3, 5, and 7, that some C group strains produced immunity in ferrets against A group. It can also be seen that the B group virus variants resembling either A or C group virus emerged from the same inoculum (Fig. 4). From these results it follows that some B and C group strains were related immunologically to the "true" distemper virus.

Fig. 8 shows in graphic form the proportion of A type virus strains compared with types B and C taken together. Assuming that the proportions in Fig. 8 are governed by the varying susceptibility of ferrets to the virus we must also accept the fact that the majority of the ferrets used had a high natural resistance or immunity to the virus, which is very improbable, and that while many ferrets appeared to be immune during passages of C strains, every ferret used was highly susceptible for the passaging of A type virus.

Regarding the suggestion that variations in the proportion of live and dead virus particles and antibody in the inocula might influence the results it seemed that this did not occur where the passaging of A type strains was concerned. The fact that diluting the inocula did not affect the difference between virus types also suggests that it is

unlikely that varying proportions of live and dead virus particles and antibody in the inocula affected the results.

The most convincing evidence, however, for the existence of virus types is the fact that the inoculation of a mixture of type A and type C virus into ferrets always resulted in the development of the A type virus only. The C type virus completely disappeared after such inoculations, and was never again recovered on subsequent passages suggesting that the more vigorous A type overgrew the slow developing C type.

The isolation of different virus types from different dogs in the same epizootic is not easy to explain, although, since viruses can readily be supposed to multiply a million-fold in one host, they are probably able to change extremely rapidly (Andrewes, 1951), and, thus, as many virus variants are possible as there are infected hosts.

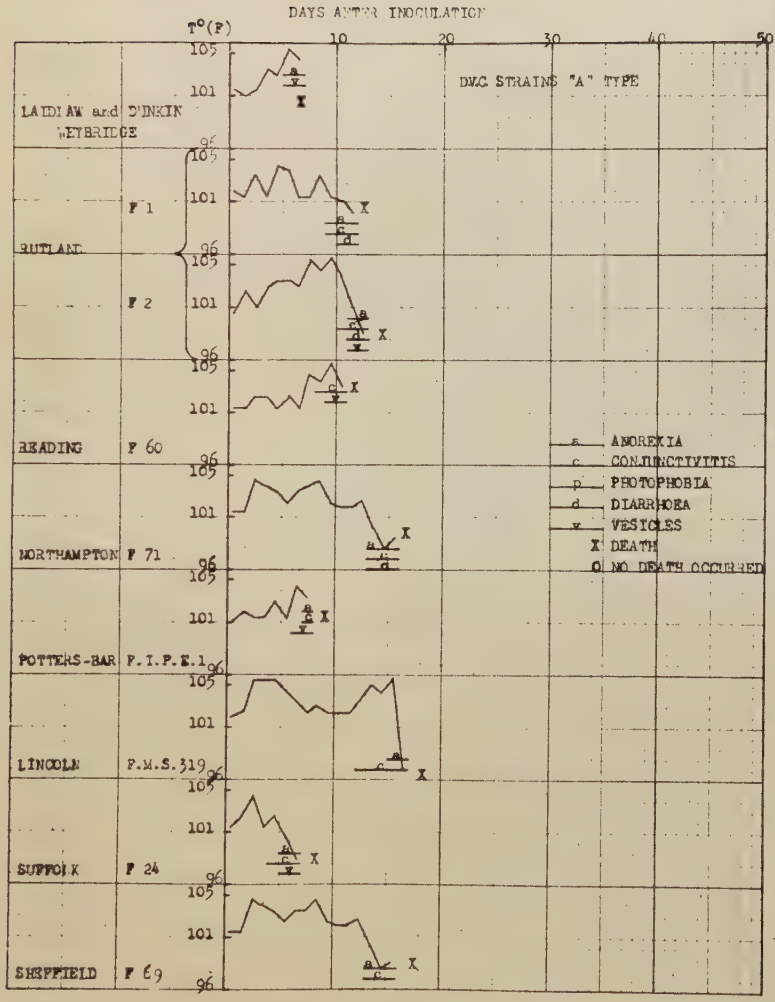
Lastly comes the question of whether the biological behaviour of virus strains in test hosts, and particularly their virulence, can be considered as a basis of virus typing, provided that the host, the inoculum, the method of inoculation, and the final effect (death in these experiments) have been specified. According to Burnet (1954) the virulence of a virus is a genetically determined function. If virulence is an inheritable quality it can obviously provide valuable material for the determining of virus change and the emergence of new types.

It is also possible that the change in the distemper virus is due to successive replacements of the A type virus by the more adaptable types B and C. In a working hypothesis to account for this phenomenon it could be postulated that (a) most virus particles carry more than one set of genetic determinants (Burnet and Lind, 1954), (b) the presence of the dominant form might determine the nature of the results, (c) the process can be very complicated if virus recombination or other genetic interactions are possible.

Further analysis of the phenomenon of variability and virus typing requires the extension and refinement of experiments. A definite decision in favour of the above, or some other hypothesis, should be possible when new and more accurate techniques are elaborated. Already it can be said that canine distemper is not caused by a homogeneous virus, as has been thought, but by several types which, far from being stable entities with predictable behaviour may change and produce new forms of a modified nature and of different biological behaviour. The phenomenon of variability can probably account for the contradictory results obtained with the same " Hard Pad " strain by MacIntyre, Montgomerie and Trevan (1948) on the one hand and Mansi (1952) on the other.

The present study of 55 virus strains has shown that their differences are sufficiently great to be taken into serious consideration in research, in diagnostic work and in the production of distemper prophylactics. In fact an appreciation of the phenomenon of virus variability demands a different approach to the problem of the diagnosis and prophylaxis of canine virus distemper.

Fig. 1.



Comparison of the behaviour in the ferret of seven A type strain-isolants, with one another, and with the "Laidlaw and Dunkin" strain.

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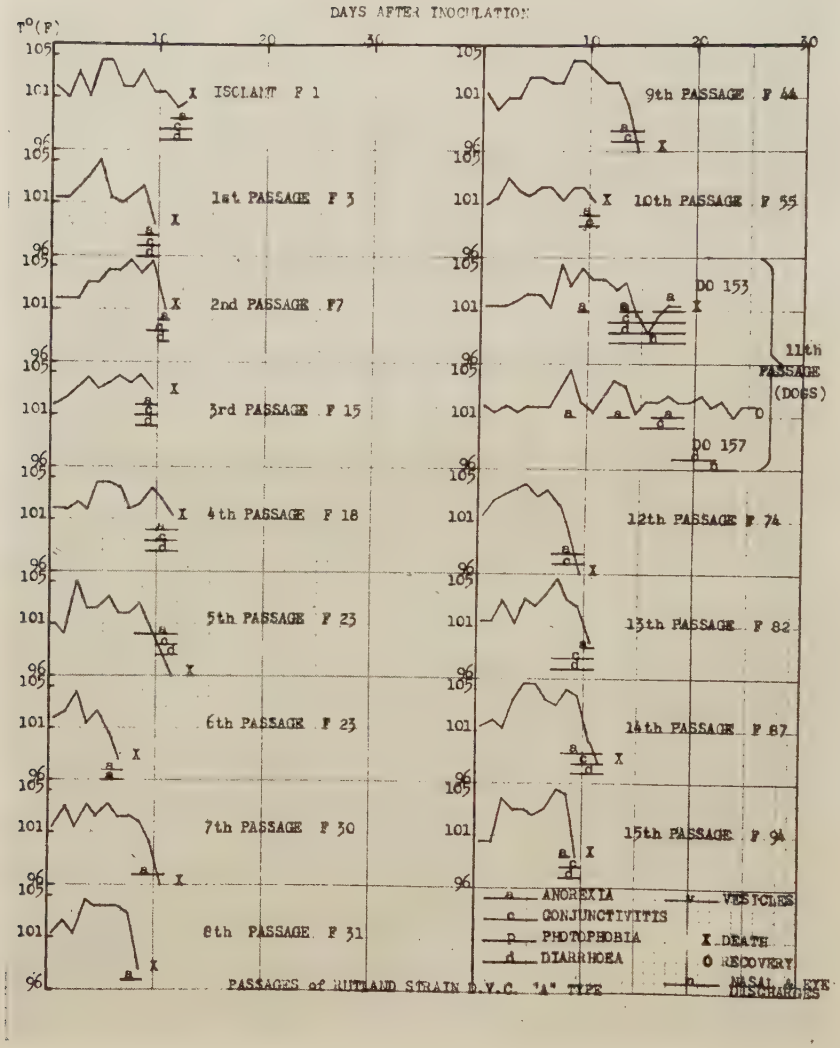
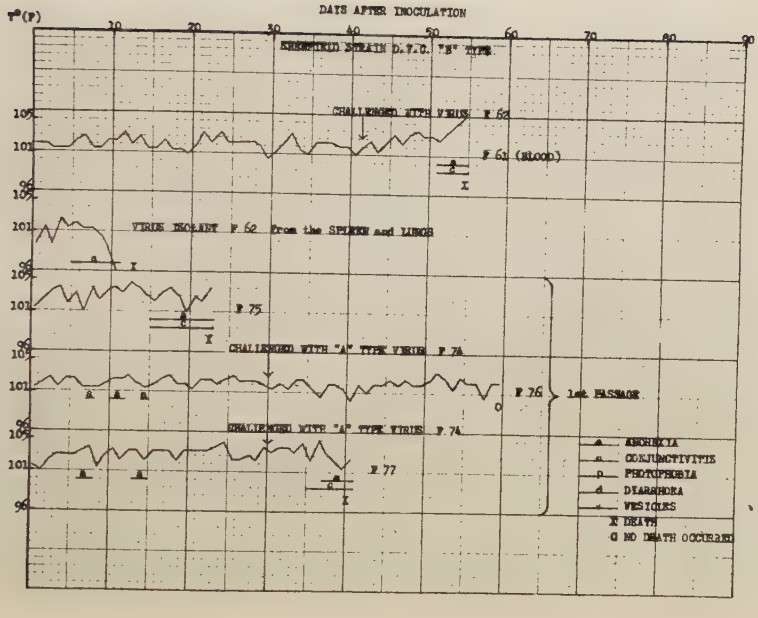
Fig. 2.



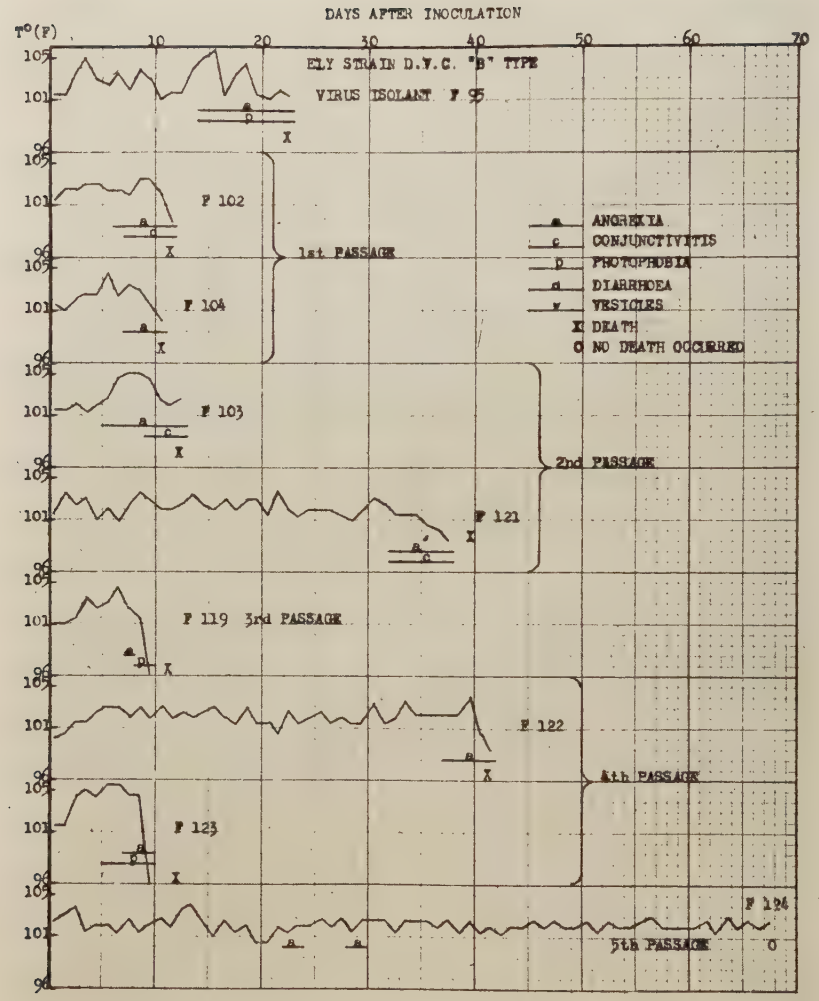
A typical experiment. The behaviour of the type A strain (Rutland) during fifteen successive passages in ferrets and dogs.

Fig. 3.



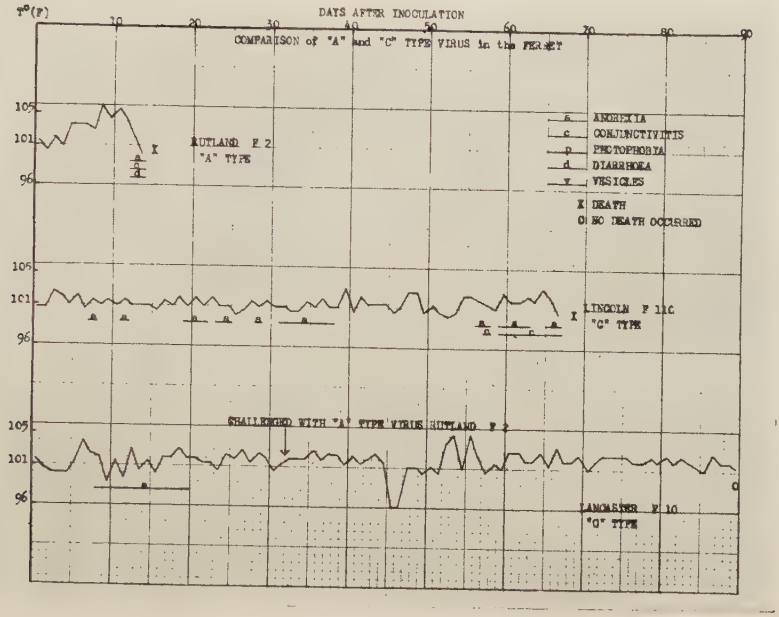
Response of the ferret to Sheffield strain B type.

Fig. 4.



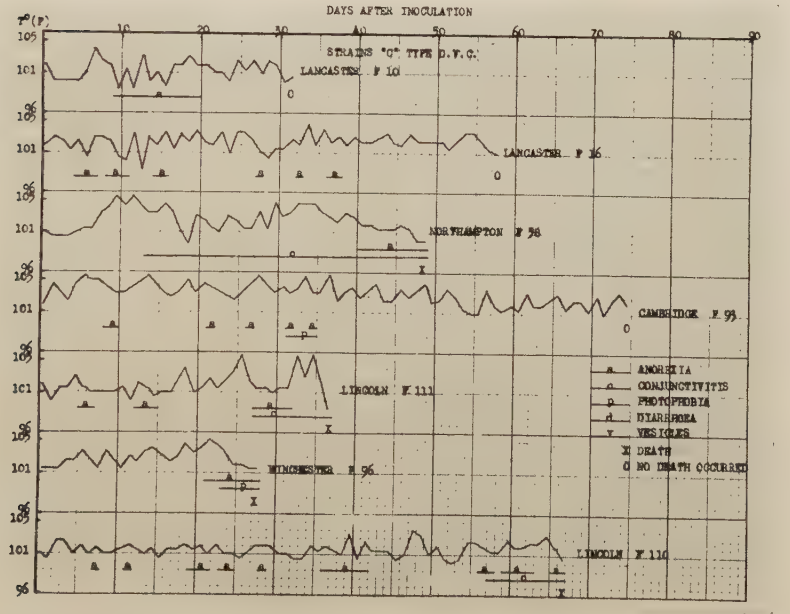
A typical example of the change in virulence for the ferret of type B strain (Ely) on successive passages.

Fig. 5.



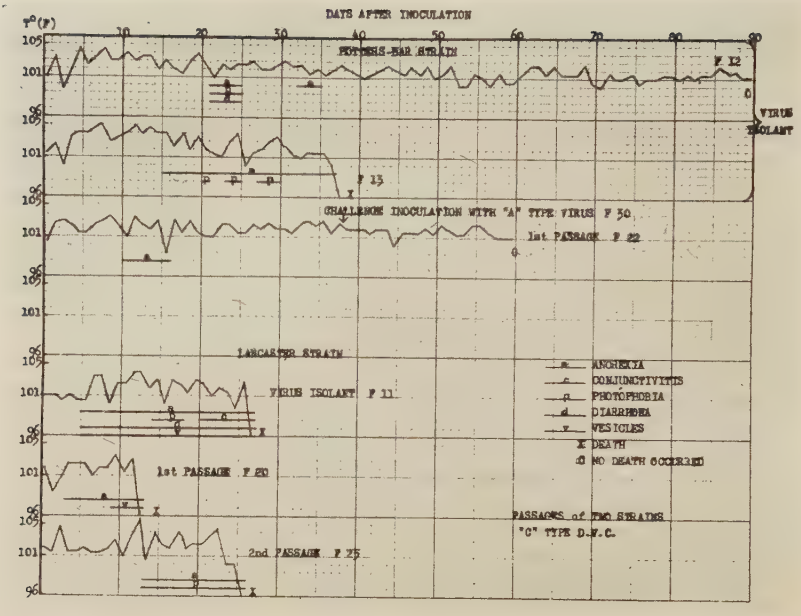
A typical example of the behaviour in the ferret of two C type strains, compared with the type A strain.

Fig. 6.



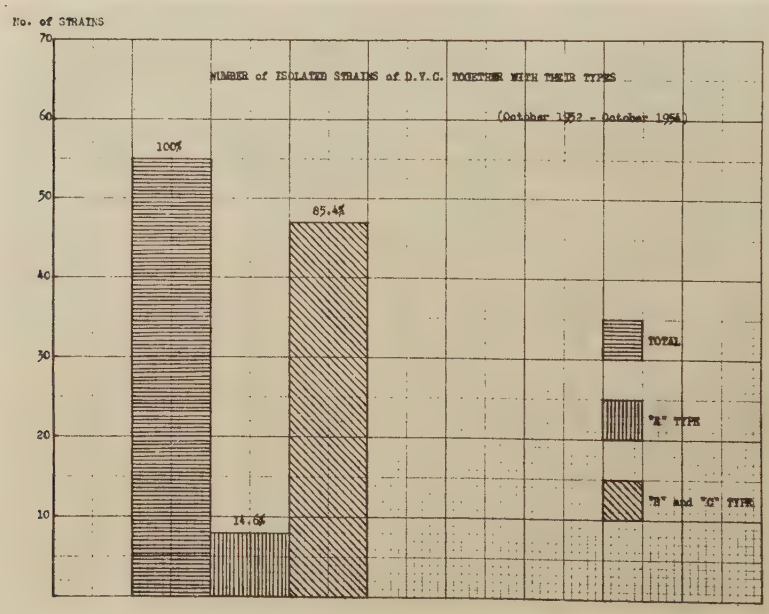
Comparison of the behaviour in the ferret of seven C type strain-isolants with one another.

Fig. 7.



A typical example of the change of two C type strains, on successive passages, in their virulence to the ferret.

Fig. 8.



Summarizing figure. The proportion of B and C type strains as compared with type A.

CONCLUSIONS

Canine distemper virus has hitherto been considered as a single entity because of its behaviour in the ferret which has been accepted as the most convenient test host for the isolation and propagation of of the virus.

The study of 55 virus strains isolated from various parts of Britain has shown, however, that the strains varied considerably on isolation and became changed in virulence on passage. Some variants of the strains studied produced no immunity in the ferret to other variants.

These findings are regarded as definite evidence that canine distemper is not caused by a homogeneous virus, but by several types which are not stable entities with predictable behaviour. These variants may undergo changes and are able to give rise to new forms of a modified nature and of different biological behaviour. The significance of this phenomenon of virus variability is briefly discussed.

ACKNOWLEDGMENTS

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STUDIES ON GRASS DISEASE

The Morphological Picture with Special Reference to the Vegetative Nervous System.

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INTRODUCTION

The fact that the post-mortem findings are but little characteristic in grass disease, has been a contributing cause to the present lack of knowledge concerning the aetiology and pathogenesis of this disease despite extensive investigation.

The various pathological changes described by different investigators are fairly uniform. In acute cases the stomach is greatly distended by gas and fluid which occasionally results in acute gastric rupture. The small intestines are also greatly distended by gas and fluid (Pool, 1928; Begg, 1936; Holman, Gordon and Pattison, 1944). Stewart, Gordon and McCallum (1940) measured the gastric and small intestinal contents in 36 cases of acute grass disease and found an average of 22 and 30 litres of fluid, respectively. In 13 normal horses the corresponding values were 7 and 9 litres, respectively. The large colon was greatly dilated and filled with compact, dry ingesta which was covered with a thin, black layer nearest the mucous membrane (Pool, 1928; Holman, *et. al.,* 1944). The mesenteric lymph nodes were swollen and contained haemorrhages (Stewart, *et al.,* 1940). According to Holman *et al.,* (1944) there were not infrequently effusions of gelatinous exudate around the oesophagus and the carotid arteries. In a few cases ulcers occur in the oesophagus. Histological examination (Holman, *et. al.,* 1944) revealed submucosal oedema and haemorrhages in the stomach and small intestines in a number of cases. In the liver there were varying changes in the form of parenchymatous degeneration, fatty infiltration, hyperaemia and cellular infiltration in the periportal spaces. In the kidneys there was nephrosis with varying changes from parenchymatous degeneration to necrosis. Hyperaemia and haemorrhages were also demonstrated in a number of cases.

Examination of the central nervous system revealed neither inflammatory changes nor inclusion bodies. At the bottom of the fourth ventricle there were small haemorrhages around the vagal and hypoglossal nuclei in a number of cases. However, this could not be regarded as significant as this also occurs in horses with other diseases. Staining the myelin material did not reveal any changes in the pons, medulla oblongata, vagus nerve, splanchnic nerve and phrenic nerve. There were no changes in the thoracic ganglion. In one case there were fat droplets in a number of ganglion cells and degeneration of isolated cells in the coeliac-mesenteric ganglion. The jugular and spinal ganglia were normal.

Haemorrhages were often demonstrable in the adrenals, especially in the cortex. In one case there was also necrosis of adrenal cortical cells. There were no changes in the hypophysis. In the cervical and mesenteric lymph glands there were oedema and haemorrhages in a number of cases. The spleen was usually slightly enlarged, apparently through hyperaemia.

The skeletal musculature and myocardium were normal.

In the chronic cases there was emaciation. The alimentary canal was of small diameter. In the stomach and the small and large intestines the contents were scanty. In the colon the contents were gruel-like and the mucous membrane was covered with large quantities of mucus. The spleen was enlarged, with a firm pulp (Pool, 1928). In a number of the chronic cases Begg (1936) also found pneumonia.

In Sweden, grass disease was first demonstrated in 1919 by Magnusson (1945) in a state horse-breeding establishment. After several years the disease disappeared, to re-appear in 1940 at a remount station. This station has since had cases of the disease each spring, with the exception of 1944. Usually it is only recently bought horses which are affected, and it only infrequently affects horses which have been at the station since the previous spring. Since 1952 cases of grass disease have occurred at another remount station.

RESULTS

Macroscopic Appearances

Post-mortem examinations were performed on 9 acute and 5 chronic cases. The macroscopic changes found were in good agreement with those of earlier investigations.

In the acute cases the stomach was greatly distended with sour, musty-smelling fluid, in advanced cases amounting to approximately 30 litres in volume. There were often many Gastrophilus larvae firmly attached to the mucous membrane. In one case, where the horse had been ill for 8 days. there were multiple pea-sized ulcers in the oesophagus. The small intestines were greatly distended with fluid and often with gas. The mucous membrane was not infrequently injected and contained many haemorrhages. The mesenteric lymph nodes were moderately enlarged, and the cut surface was moist. In the caecum and the first part of the colon the contents were abundant and of a rather firm consistency. In the third and fourth parts of the colon there was generally more or less advanced coprostasis with hard, compact, dry contents. In severe cases there was coprostasis even in the first or second parts of the colon. In severe cases the mucous membrane was firmly adherent to the ingesta, which was dark coloured next to the mucosa. However, in one case the contents of the large intestine were normal. The small colon contained relatively small faecal boluses covered with mucus. In one case there was an acute necrotic colitis in the anterior part of the small colon.

The lungs, heart, liver and kidneys showed no macroscopic changes. The adrenals were usually moderately enlarged and in several cases there were recent haemorrhages in the cortex.

In some cases punctate haemorrhages were demonstrated along the vagus and sympathetic nerves in the cervical region and along the sympathetic trunk in the thorax. In the brain and spinal cord no noteworthy changes were observed.

In the chronic cases, some 3 weeks to 2 months after the horse had become ill, the carcase was emaciated and dehydrated, with advanced muscular atrophy. In contrast to the acute cases, the stomach was very small and often contained only about two to three litres of half-liquid, gruel-like ingesta. The contents of the small intestine were also scanty and the mucous membrane appeared normal. In three cases approximately two metres of the last part of the ileum were dilated and moderate muscular hypertrophy was present. The caecum and the large colon were of small diameter and had only small quantities of semi-solid contents. As in the acute cases, there were large and small verminous aneurysms in the mesenteric arteries. The pancreas was atrophic and of firm consistency. The adrenals were enlarged and had a widened cortex. The heart, lungs, liver and kidneys showed no noteworthy changes.

Microscopic Appearances

The clinical picture of this disease is strongly indicative of a disturbance in the vegetative nervous system with sympathicotonia (Greig, 1928, 1942). Therefore the vegetative nervous system was dissected out in all cases and histological examinations performed on the sympathetic trunk, the prevertebral ganglia, the vagal-glossopharyngeal nucleus in the medulla oblongata, the plexus nodosus and the spinal cord. In addition, parts of the cerebrum and cerebellum and the spinal ganglia were examined. With the exception of 2 cases, where autopsy was performed 6 and 3 hours, respectively, after death, the material was fixed within half an hour of death.

Nervous System

Acute cases

Advanced changes were demonstrated in the ganglion cells in the sympathetic ganglia (the cranial cervical ganglion, the stellate ganglion, the thoracic and abdominal sympathetic trunk, the solar ganglion and the caudal mesenteric ganglion).

In horses killed one or more days after the occurrence of symptoms, the majority of ganglion cells were swollen and had smooth outer contours. They were the site of different stages of chromatolysis. The Nissl substance disappeared first in the centre of the cells, but was retained in a small zone at the periphery of the cells, later disappearing even from this region. The nuclei lay eccentrically and the nucleolus was enlarged and in common with the nuclear membrane, strongly basophilic. Otherwise the nucleus appeared deficient in chromatin (Fig. 3).

After a further period of illness small vacuoles occurred in the cytoplasm of some of the chromatolytic cells, first singly and then in increasing numbers, so that the cells appeared spongy and swollen (Fig. 7). The vacuoles often lay at the centre of the cell and in a peripheral zone, with retention of a layer of condensed, eosinophilic

cytoplasm between these zones. In some cells many such zones could alternate, or there could be vacuolation either centrally or peripherally in the cell (Fig. 8). The processes of the neurons were also enlarged and contained vacuoles. A number of the chromatolytic cells were not immediately subject to vacuolation but shrank with the cytoplasm becoming condensed and more basophilic. Similar vacuoles to those mentioned above also occurred in these cells (Fig. 6). There was often karyopyknosis and karyolysis of the nucleus when these vacuoles occurred (Fig. 8). In the nuclei of normal or slightly chromatolytic cells there were enlarged nucleoli and a small zone of densely stained nucleoproteids along the nuclear membrane, so-called nuclear caps (Fig. 9).

In the cytoplasm of both affected and normal ganglion cells there were eosinophilic granules resembling inclusion bodies. These were best demonstrated by phloxine staining, when they were stained a bright red. They usually lay in groups at the periphery of the cell, often near collections of pigment. The granules could also occur throughout the whole cytoplasm. These structures, which resemble those described by Stenius (1952) in bovine malignant catarrh, have, however, been demonstrated in normal horses, so that they were of no importance in this connection. They were most numerous in the sympathetic ganglion cells, but also occurred in the medulla oblongata, the spinal cord and the spinal ganglia.

Silver impregnation only slightly stained the chromatolytic cells (Fig. 11). In the other neurons there were occasionally " Fortsatzdisharmonie " (Stöhr, 1943), i.e. one or more of the processes of the neurons were abnormally wide (Fig. 12). These are regarded as defects of the neurons and are not uncommon in horses suffering from various diseases (Köhler, 1952). Another characteristic phenomenon in grass disease is deposit of hyalinoid substances in the cytoplasm of the ganglion cells. These can be observed as spherical, dense, weakly eosinophilic and strongly silver positive, homogeneous masses usually demarcated from the cytoplasm by a thin space (Fig. 10). This change can also be found both in otherwise unchanged cells and in chromatolytic ganglion cells. Köhler (1952), who observed this change in the horse but not in other species, regarded this as a reaction typical to the horse. In individual cells there was also so-called " window formation," i.e. vacuoles near the cell membrane.

In rare cases there were neutrophil leukocytes surrounding extensively vacuolated ganglion cells, but there were otherwise no inflammatory cell elements. In the horse there are normally small collections of lymphocytes in the ganglia, but these showed no signs of activation. No proliferation of the capsular cells was found in acute cases despite often severe damage to the neurons.

The most marked changes were found in the cranial cervical ganglion, the stellate ganglion and the sympathetic trunk, while the solar and caudal mesenteric ganglia were often affected to a lesser

extent. Examination of the intramural ganglia in the gastric and intestinal wall revealed that the cells in Meissner's plexus showed only insignificant damage, chiefly chromatolysis. In Auerbach's plexus the changes were more marked. Here there were numerous chromatolytic, often extensively vacuolated cells (Fig. 20), and basophilic, shrunken cells (Fig. 21). In the vagal-glossopharyngeal nucleus there were no changes. In the hypoglossal nucleus there was in one case wide-spread chromatolysis of the ganglion cells and shrinking and basophilia of a number of them (Fig. 22).

The majority of ganglion cells in the plexus nodosus had a normal appearance. Individual cells, however, showed chromatolysis, commonly with peripheral retention of Nissl substance (Fig. 23).

In the posterior part of the spinal cord numerous ganglion cells in the lateral horn and a few ganglion cells in the ventral horn were chromatolytic and vacuolated. These cells usually lacked nuclei. There were somewhat more severe changes in the spinal ganglia than in the plexus nodosus. The larger ganglion cells were often undamaged while a number of the smaller ones were chromatolytic, often with retention of a small peripheral zone of Nissl substance (Fig. 24). A number of the chromatolytic ganglion cells were subject to shrinking and basophilia, and in some of them there was also vacuolation.

In the cerebrum, cerebellum and the brain stem (except in the hypoglossal nucleus in one case) no changes were observed.

Chronic cases

In the chronic cases the damage to the ganglion cells was as extensive as in the acute cases. The sympathetic ganglia were markedly deficient in morphologically normal ganglion cells. In a number of cells with central chromatolysis the remaining peripheral Nissl substance had condensed to coarser, densely staining masses (Fig. 16). A number of cells had a border of coarse Nissl substance along the nuclear membrane (Fig. 15). Individual ganglion cells were shrunken with extreme chromophilia. There were overall numerous, extensively vacuolated, weakly staining cell shadows or markedly shrunken, eosinophilic clumps. The cell remnants appeared to be destroyed by autolysis. In the majority of cases resorption is performed without reaction by the capsular cells. Local proliferation of capsular cells did sometimes occur at the site of a neuron which had entirely disappeared, ((" Restknötchen," according to Nageotte) (Fig. 14). In Auerbach's plexus there were extensively vacuolated ganglion cell remains and hyperchromatic, shrunken ganglion cells. A number of chromatolytic ganglion cells contained coarse Nissl substance around the nuclear membrane. In Meissner's plexus no changes were observed. In the plexus nodosus and the spinal ganglia there were isolated chromatolytic ganglion cells with condensed Nissl substance at the periphery. A number of necrotic ganglion cells lay as eosinophilic débris, often surrounded by proliferating capsular

cells. Chromophilic, shrunken ganglion cells, usually with pyknotic nuclei, were also present. The changes were less extensive in the plexus nodosus than in the spinal ganglia. In the posterior spinal cord the ventral and lateral horns contained individual necrotic nerve cells without nuclei, and extensively vacuolated or formed eosinophilic, compact clumps.

No changes were observed in the other parts of the central nervous system.

Other Organs

In acute cases histological examination of the gastric and intestinal mucous membrane generally revealed no noteworthy changes with the exception of the haemorrhages already mentioned in the mucosa.

In the colonic mucous membrane the crypts were filled with thick, eosinophilic plugs of secretion. The mucous membrane was covered by a layer of mucus mixed with ingesta and detached epithelial cells. This layer can be shown to contain haemoglobin by Lepehne's haemoglobin stain and haemosiderin by Hueck's modified Tirmann-Schmelzer method. In one case there was an acute catarrhal-purulent colitis with numerous leukocytes in the crypts. In the acute cases, there was generally an irregularly distributed, simple fatty infiltration in the liver. In the chronic cases there was usually a slight perilobular infiltration of lymphocytes.

The pancreas in the acute cases appeared normal. After 4 days there seemed to be a diminution of secretory granules. In the chronic cases, in contrast, there was marked atrophy of the exocrine glandular cells. These were basophilic and contained secretory granules.

Similar conditions ruled in the parotid gland. After several days of illness there was marked reduction of secretory granules and these were present only in single, or in groups of, acini. The cell nuclei had migrated to the centre of the cells. The same picture was observed in the sub-acute stage (2 cases, ill 2 and 15 days, respectively). Similar changes can be produced experimentally by stimulation of the sympathetic supply to the parotid (Best and Taylor, 1950).

In acute cases there was generally mild nephrosis. The epithelial cells in the convoluted tubules and in Henle's loop showed individual degenerative nuclear changes, such as karyopyknosis and karyolysis. The lumina were dilated and contained weakly eosinophilic floccular masses or homogeneous cylinders. In the chronic cases no renal changes were observed.

In the myocardium foci of slight, acute alterative myocarditis were observed in one case. In all the other cases the histological examination was negative in this respect. In the skeletal musculature a slight acute waxy degeneration of the muscle fibres was observed in two cases. In the other cases there were no changes.

In the earliest case, in which the horse was killed after only a few hours of illness, the adrenals showed no changes. In the other acute cases there was a more or less marked widening of the cortex and hyperplasia of the zona arcuata. The lipoid content was reduced, occasionally to the extent of complete disappearance from the cortex. Fresh haemorrhages in the zona arcuata and the neighbouring part of the zona fasciculata could be present. In connection with this change the epithelial cells were usually dissociated and subject to necrobiotic and necrotic changes. In sub-acute and chronic cases similar changes were observed, but commonly to a greater extent. In one of the sub-acute cases the haemorrhage in the cortex was localised to the zona reticularis. The changes observed could be expression of a stress reaction (Selye, 1949). The hypophysis showed no definite variations from the normal picture in both acute and chronic cases.

DISCUSSION

The most fundamental changes in grass disease are localised in the sympathetic ganglia. Knowledge of the normal variations in ganglion cell structure during different stages of cell activity is of great value in understanding these changes.

Using the method of Caspersson (1950) with absorption analysis in ultraviolet light, Hydén (1943) carried out valuable experiments on this subject. He studied ganglion cells in different functional stages and found that the Nissl bodies which largely consist of ribose nucleotides (Caspersson, Landström and Wohlfart, 1941) are greatly reduced in the motor neurons during activity. In connection with this, certain reactions occur in the nucleus to compensate for the Nissl substance utilised. The nucleolus becomes enlarged and acquires an increased content of ribose nucleotides. An accumulation of heterochromatin, " nucleolar associated chromatin," appears around the nucleolus. The nucleus becomes increasingly eccentrically located in the cell and the nuclear membrane becomes more basophilic due to an increased content of chromatin. From the nucleolus a stream of proteins, rich in hexone bases, is directed towards the nuclear membrane. At the nuclear membrane synthesis of cytoplasmic protein containing ribose nucleotides occurs.

The changes in the ganglion cells in grass disease resemble to some extent the changes in poliomyelitis in man. In the initial stage of the latter disease there are signs of an intensive stimulation of the cytoplasmic protein forming system in the neurons (Caspersson, 1950). The nucleus increases in volume, the nucleolus becomes greatly enlarged and the chromatin is collected along the nuclear membrane. First increasing amounts of ribose nucleotides are observed along the nuclear membrane and then the capacity of the cell to synthesize new protein ceases and the ribose nucleotides disappear rapidly in the cytoplasm, beginning nearest the nucleus. Usually a zone of ribose nucleotides is retained peripherally in the

cell. In this stage the nucleus is filled with granules which strongly absorb ultraviolet light. Haematoxylin-eosin staining reveals intranuclear eosinophilic inclusions (Bodian, 1949). The most damaged cells then die. The nucleus shrinks and breaks up and the cytoplasm becomes vacuolated.

When the acute stage of the disease is over, a number of the chromatolytic cells are restored and ribose nucleotides are again deposited in the cytoplasm, beginning nearest the nuclear membrane (nuclear caps). A number of these cells acquire an abnormally high content of ribose nucleotides. These chromophilous cells have lost their impulse activity (Einarson, 1949).

The appearance of the nucleus in the first stage of grass disease, when only chromatolysis is present, shows that there is also a strong stimulation to protein synthesis. In many cases, however, the stimulation does not result in protein synthesis but the cell dies and the nucleus suffers irreversible degenerative changes. Small vacuoles occur in the cytoplasm at an early stage when only chromatolysis is present, without any degenerative changes in the nucleus. This vacuolation should be an irreversible process (Zollinger, 1948). In those nerve cells, where the cell damage continues to necrosis, these vacuoles become enlarged and the cell is completely destroyed. Even a few days after the beginning of grass disease there are indications that a number of ganglion cells again synthesize cytoplasmic protein in the form of " nuclear caps," i.e. accumulations of ribose nucleotides at the nuclear membrane.

In the chronic cases many of the living ganglion cells have large, presumably newly-formed, Nissl bodies sparsely distributed, often accumulated along the nuclear membrane. These cells have presumably been chromatolysed in the acute stage, but have since been again able to synthesize cytoplasmic nucleotides. A similar picture can be produced experimentally by section of a motor nerve. In the associated ganglion cells, which first become chromatolysed similar coarse Nissl bodies appear in the restitution stage (Hydén, 1943). Another typical finding in the chronic cases of grass disease are nerve cells with central chromatolysis and condensed Nissl bodies at the periphery. This cell picture is also common in chronic cases of poliomyelitis. Bodian (1949) regarded these Nissl bodies as newly synthesized. This is, however, improbable, first as these cells often have severe degenerative changes in the nuclei and secondly as the Nissl substance is formed only at the nuclear membrane (Hydén, 1943).

Hyperchromatic ganglion cells, which are common in poliomyelitis and also occur in grass disease should, according to Einarson, (1949) be formed by chromatolytic cells which have again become able to form nucleotides but which have not re-acquired impulse activity. These cells will eventually be able to function normally or else they will die, with continued diminution and disappearance of the Nissl substance.

As is apparent the nerve cell damage in grass disease corresponds to a great extent with the changes in poliomyelitis. The chief difference between these two diseases, apart from the localisation of the changes, is the occurrence of inflammatory changes in poliomyelitis in which it is considered that the virus stimulates protein synthesis in order to change the substances formed to virus protein at a certain stage of the synthesis (Caspersson, 1950). If similar conditions exist in grass disease it could thus be expected that the affected ganglia would have ceased to function and not show increased activity. Both the clinical picture and the cell picture in the acute stage indicate that the agent causing grass disease stimulates the cells to such high impulse activity that total chromatolysis often with cell death is the result.

Greig (1928) considered that the clinical symptoms in grass disease were an expression of sympathicotonia. The only symptom lacking was contraction of the pupil. He assumed that certain amino bases, tyramine and phenylethylamine, would be released by incomplete breakdown of amino acids. These substances cause sympathetic stimulation. The disturbance could be primarily caused by some substance (toxin or virus) which could inhibit the normal breakdown of the above amino-bases in the liver. In a later article Greig (1942) considered that grass disease was caused by a neurotropic virus. He believed that the disease was probably not transmitted by contact and that the infective agent was presumably widely distributed, but that only certain individuals were susceptible. He pointed out several similarities to poliomyelitis which is also seasonal and often occurs with several cases in a certain area.

Other theories as to the cause of grass disease have all been abandoned after continued investigation. Tocher (1923) considered that it was botulism as he succeeded in isolating a bacterium resembling *Clostridium botulinum* from cases of grass disease. Walker (1929) who administered botulinus toxin to experimental horses, produced paralysis of the tongue and pharynx but none of the other symptoms of grass disease.

Gordon (1934) isolated a toxin from *Cl. welchii*, type D from the intestinal contents of horses with grass disease. He considered this the cause of the disease. A later immunisation experiment of 972 horses with type D toxin did not prevent grass disease.

Analysis of soil from pastures for soil type, calcium, phosphorus and nitrogen revealed no deficiencies (Stewart, 1941). Poisonous plants which could explain the occurrence of the disease (Guthrie, 1940; Magnusson, 1945) could not be demonstrated on the pastures.

It thus appears that Greig's conception of the disease is the only one which so far has not been refuted. His view that sympathicotonia is present is well confirmed by the changes observed in the sympathetic ganglia. He has not, however, been able to present any conclusive evidence of the cause of the increased sympathetic activity.

In grass disease only purely regressive changes have been

observed in the ganglia and no inflammatory changes. This would appear to be additional evidence of intoxication rather than of a virus disease. There is, however, one example of a neurotropic virus producing only regressive changes, viz. scrapie. This sheep disease is considered to be due to a transmissible, filterable virus with a long period of incubation. Wagner, Goldstein, Doran and Hay (1954) found histological changes in the pons, medulla oblongata and cervical spinal cord in scrapie, comparable with the picture in grass disease. The ganglion cells showed disintegration of Nissl substance and vacuolation. Cytoplasmic or intranuclear inclusion bodies, which were stained by haematoxylin-eosin or with methylene blue, were demonstrable. In some sections a slight gliosis without an inflammatory reaction was seen. In respect to poliomyelitis Einarson (1949) and Bodian (1949) considered that the degenerative changes in the nerve cells were primary and did not necessarily have to be followed by inflammatory changes.

In domestic animals no changes have been described previously in the vegetative nervous system which are comparable with those in grass disease. In man and animals a number of regressive changes and proliferative processes have, of course, been described in the vegetative nervous system, but these are more non-specific damage and have nothing in common with grass disease. They occur in man in clinically healthy individuals and in patients with various symptoms of disturbances in the vegetative nervous system (bronchial asthma, stomach ulcer, Raynaud's disease), although the frequency of such changes is considerably higher in the latter conditions. In the horse, v. Hein (1952) and Köhler and v. Hein (1953) investigated the intramural ganglia in colic and found a markedly higher number of changed cells in colic than in normal slaughtered horses. They considered that the cell damage was a result of the attack of colic and not the cause of the colic. Similar damage in ganglion cells has also been observed in grass disease, but the affected cells are not more numerous than can be considered normal.

The changes found in the ganglion cells of the vegetative nervous system in grass disease explain the severe clinical course with marked resistance to all therapy. They resemble those caused by neurotropic viruses. Insufficient is known of the way in which nerve cells react to different agents to draw definite conclusions as to the aetiology. The possibility of intoxication can, therefore, not be excluded, especially as grass disease has, as yet, not been transmitted.

CONCLUSIONS

The author has examined 9 acute and 5 chronic cases of grass disease in horses. The macroscopic changes observed correspond to those found by previous investigators.

In the histological investigation of the vertebral and prevertebral ganglia extensive changes were found in the acute stage. The majority of the ganglion cells were at first chromatolytic and later

displayed pyknotic nuclei and vacuolation of the cytoplasm. Similar changes were found in Auerbach's plexus, but only slight changes in Meissner's plexus. In the vagal-glossopharyngeal nuclei in the medulla oblongata no changes were observed, but in one case there was extensive chromatolysis in the hypoglossal nucleus. In the lateral and ventral horns of the spinal cord, the plexus nodosus and the spinal ganglia there was occasional chromatolysis and vacuolation of the nerve cells.

In the chronic cases the sympathetic ganglia contained very few living ganglion cells but numerous vacuolated cell ghosts and chromophilic shrunken cells. Some of the chromatolytic cells had an accumulation of Nissl substance at the nuclear membrane. In otherwise normal ganglion cells there were often coarse, sparsely distributed Nissl bodies. Similar changes were found in Auerbach's plexus. In the spinal cord occasional necrotic nerve cells were seen in the ventral and lateral horns, with similar changes appearing in the plexus nodosus and the spinal ganglia.

The nerve cell damage in the vegetative nervous system in grass disease is compatible with the sympathicotonia observed clinically and explains the poor prognosis of the disease. Although similar changes are caused by neurotropic viruses, e.g. poliomyelitis, this does not exclude the possibility of a toxic aetiology.

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LEGENDS TO ILLUSTRATIONS.

Fig. 1. The caudal cervical ganglion of a normal horse. Haemalum and eosin. 500x.

Fig. 2. A ganglion cell from the caudal cervical ganglion of a normal horse. Haemalum and eosin. 1125 x.

Fig. 3. O 3692/54. Acute grass disease. Died on the third day of illness. Ganglion from the thoracic part of the sympathetic trunk. The majority of the ganglion cells are enlarged with rounded borders and subject to chromatolysis. Only a small zone of Nissl substance is visible peripherally. The nuclei are also enlarged and contain enlarged nucleoli. At N there is a normal ganglion cell. Haemalum and eosin. 300 x.

Fig. 4. O 3832/53. Acute grass disease. Killed on the first day of illness. Plexus hypogastricus. At C there is a chromatolytic cell with peripheral retention of Nissl substance, and at B a chromatolytic, basophilic, shrunken ganglion cell. Haemalum and eosin. 500 x.

Fig. 5. O 3692/54. Acute grass diease. Killed on the third day of illness. Ganglion cell from the thoracic part of the sympathetic trunk. Marked chromatolysis with peripheral retention of Nissl substance. Small vacuoles are present in the cytoplasm at the centre of the cell. The nucleolus is enlarged and the nuclear membrane is more basophilic. Haemalum and eosin. 1125 x.

Fig. 6. O 3692/54. Acute grass disease. (Same case as in Fig. 5). Ganglion cell from the cranial cervical ganglion. The markedly chromatolytic ganglion cell is shrunken and basophilic. Small vacuoles are present in the cytoplasm. Haemalum and eosin. 1125 x.

Fig. 7. O 3412/54. Sub-acute grass disease. Killed 12 days after becoming ill. Caudal cervical ganglion. The ganglion cells in the initial stages of chromatolysis have become vacuolated to a large extent and have acquired pyknotic nuclei. Haemalum and eosin. 300 x.

Fig. 8. O 3412/54. Sub-acute grass disease. (Some case as in Fig. 7). Ganglion cell from the cranial cervical ganglion. Total chromatolysis of the cytoplasm. Vacuoles in the cytoplasm form concentric zones. The nucleus is pyknotic. Haemalum and eosin. 1125 x.

Fig. 9. O 3412/54. Sub-acute grass disease. (Same case as in Fig. 7). Ganglion cell from the cranial cervical ganglion. The cytoplasm is weakly chromatolytic. The nucleus shows the picture of a cell in an active stage of protein synthesis. At the lower border of the nucleus is an accumulation of ribose nucleotides, a so-called nuclear cap, beside the nuclear membrane. Haemalum and eosin. 1125 x.

Fig. 10. O 3410/54. Acute grass disease. Killed on the third day of illness. Solar ganglion. Numerous chromatolytic, extensively vacuolated ganglion cells. At H there are hyaline globules present in otherwise normal ganglion cells. Einarson's gallocyanin-chrome alum method. 300 x.

Fig. 11. O 3632/53. Acute grass disease. Killed on the first day of illness. A ganglion from the thoracic part of the sympathetic trunk. The chromatolytic ganglion cells (C) are not argyrophile but have normal staining properties in the processes. Silver staining according to Bielschowsky-Gros. 300 x.

Fig. 12. O 4103/54. Chronic grass disease. (Killed when moribund after 36 days illness). Cranial cervical ganglion. Hypertrophy of one process (" Fortsatzdisharmonie"). Silver stain according to Palmgren. 500 x.

Fig. 13. O 4282/53. Chronic grass disease. Killed in extremis 5 weeks after becoming ill. Cranial cervical ganglion. Only occasional normal ganglion cells remain (N). At V there are extensively vacuolated remnants of ganglion cells. At B there is a shrunken basophilic ganglion cell without Nissl substance.
Haemalum and eosin. 300 x.

Fig. 14. O 4414/53. Chronic grass disease. Died after 5 weeks' illness. Hypogastric plexus. At R there are proliferating capsular cells where there had previously been a ganglion cell (" Restknôtchen ").
Einarson's gallocyanin-chrome alum method. 300 x.

Fig. 15. O 4282/53. Chronic grass disease. Killed in extremis 5 weeks after becoming ill. Stellate ganglion. At C there are chromatolytic ganglion cells with condensed peripheral Nissl bodies. At N there is a ganglion cell with newly-formed coarse Nissl bodies in the centre of the cell.
Einarson's gallocyanin-chrome alum method. 500 x.

Fig. 16. O 4282/53. Chronic grass disease. (Same case as i Fig. 15.). Stellate ganglion. The ganglion cell to the right is chromatolytic. Beside the nuclear membrane there is an accumulation of newly-formed ribose nucleotides. The ganglion cell to the left is chromatolytic with condensed Nissl substance at the periphery.
Haemalum and eosin. 1125 x.

Fig. 17. O 4412/53. Chronic grass disease. Died after 5 weeks' illness. Stellate ganglion. Many of the relatively normal cells contain coarse, sparsely distributed, presumably newly-formed Nissl bodies.
Haemalum and eosin. 500 x.

Fig. 18. O 4282/53. Chronic grass disease. Killed in extremis 5 weeks after becoming ill. Solar ganglion. Ganglion cell which had presumably previously been chromatolytic. The nucleolus is enlarged and surrounded by nucleolar-associated chromatin. The chromatin lies grouped round the nuclear membrane. Coarse, newly-formed Nissl bodies in the cytoplasm. (Compare with Fig. 2.).
Haemalum and eosin. 1125 x.

Fig. 19. O 3412/54. Sub-acute grass disease. Killed 12 days after becoming ill. Cranial cervical ganglion. Ganglion cell, subject to chromophilia and shrinking.
Haemalum and eosin. 1125 x.

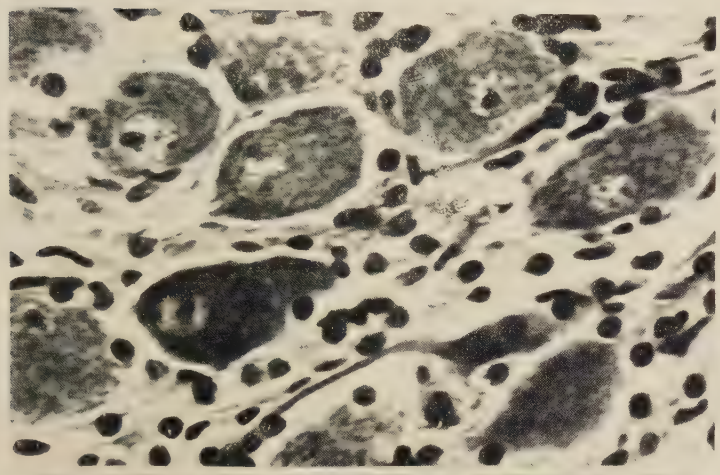
Fig. 20. O 3411/54. Acute grass disease. Died on the third day of illness. Small intestine. Auerbach's plexus. At C there is a chromatolytic vacuolated ganglion cell. At N there is a normal ganglion cell. Einarson's gallocyanin-chrome alum method. 500 x.

Fig. 21. O 3692/54. Acute grass disease. Killed on the third day of illness. Small intestine. Shrunken, basophilic ganglion cell from Auerbach's plexus.
Haemalum and eosin. 1125 x.

Fig. 22. O 3412/53. Subacute grass disease. Killed 12 days after becoming ill. The hypoglossal nucleus. The majority of the nerve cells are subject to chromatolysis (C) and a number of these are also subject to shrinking and basophilia (B). At N there is a normal ganglion cell.
Einarson's gallocyanin-chrome alum method. 150 x.

Fig. 23. O 3412/54. Sub-acute grass disease. Killed 12 days after becoming ill. Plexus nodosus. Occasional chromatolytic ganglion cells are present ; some of these show commencing vacuolation (V).
Einarson's gallocyanin-chrome alum method. 300 x.

Fig. 24. O 3727/54. Acute grass disease. Killed on the fourth day of illness. Spinal ganglion. A number of ganglion cells (C) are chromatolytic with the peripheral retention of Nissl substance. Einarson's gallocyanin-chrome alum method. 300 x.



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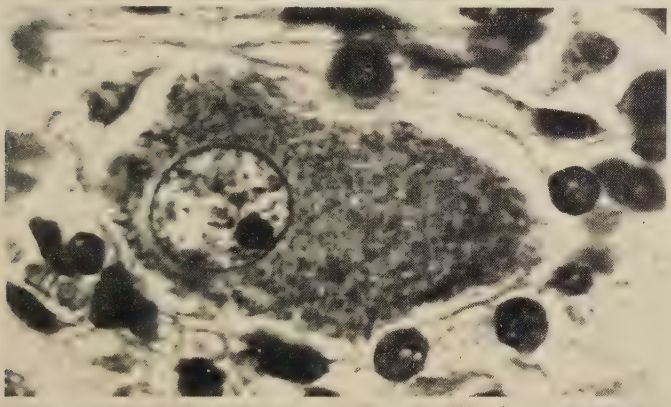

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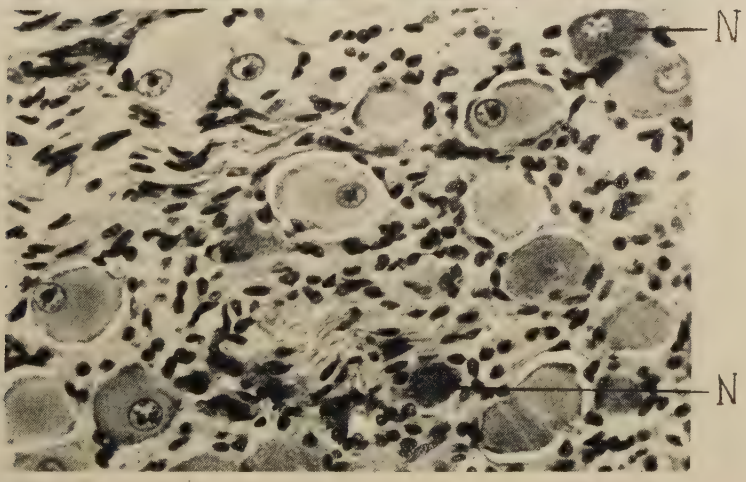

Fig.3

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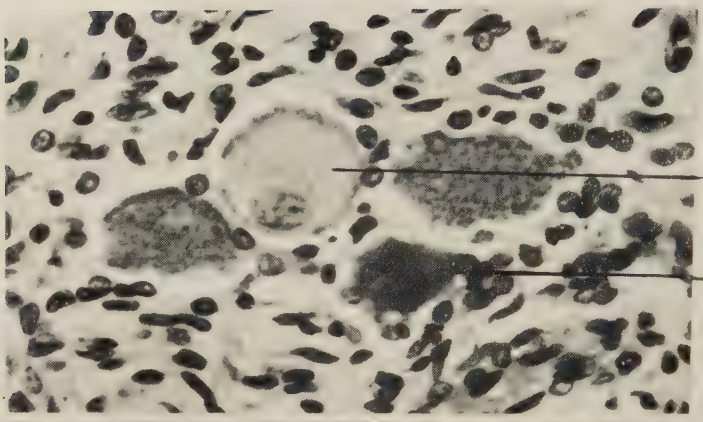

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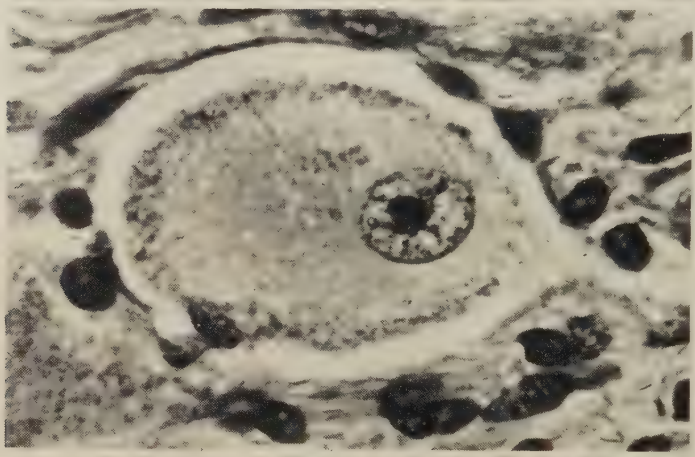

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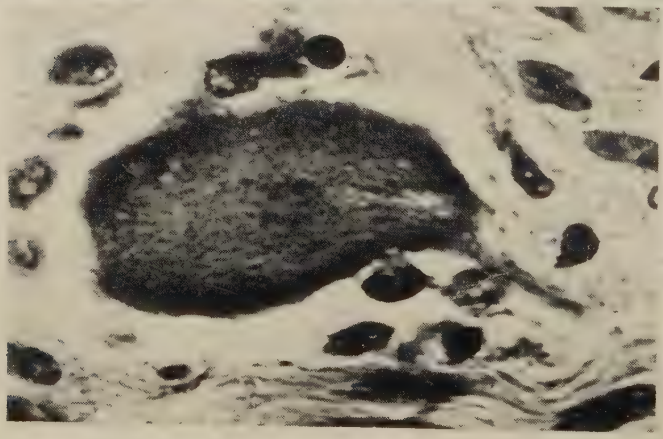

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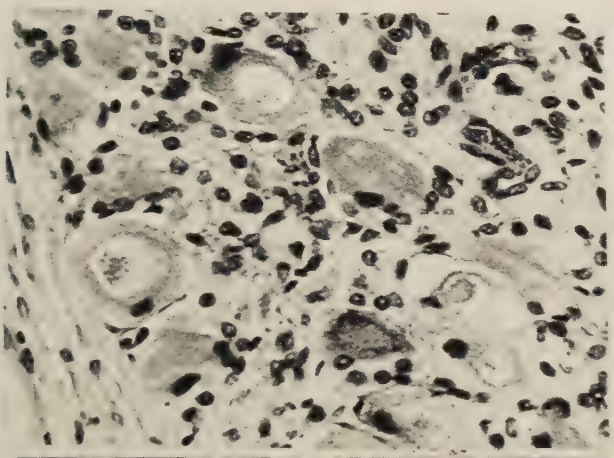

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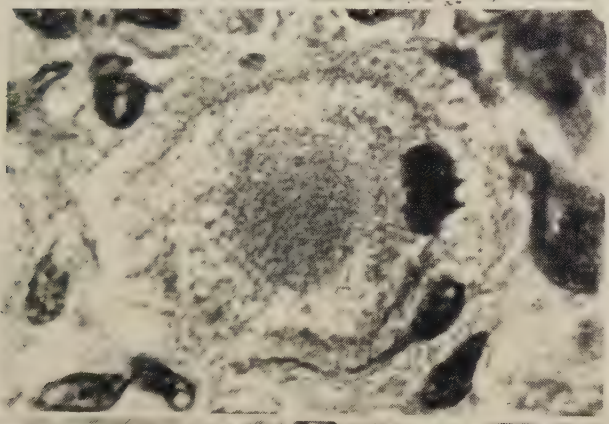

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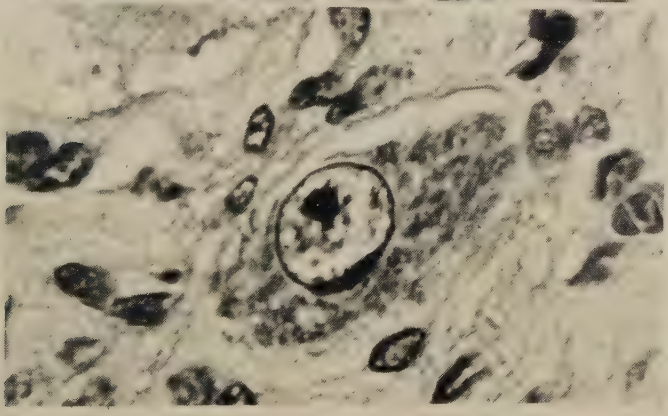

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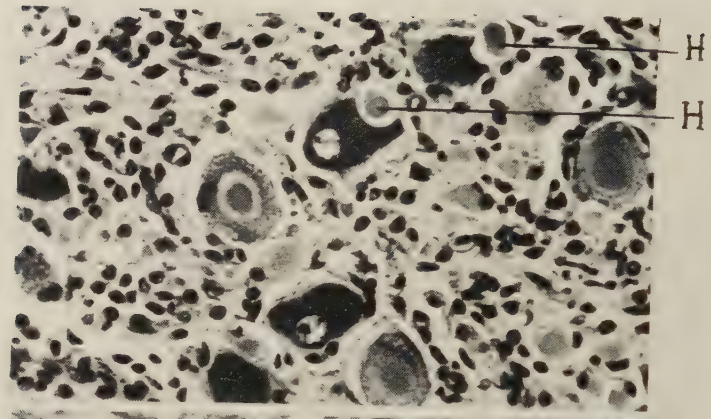

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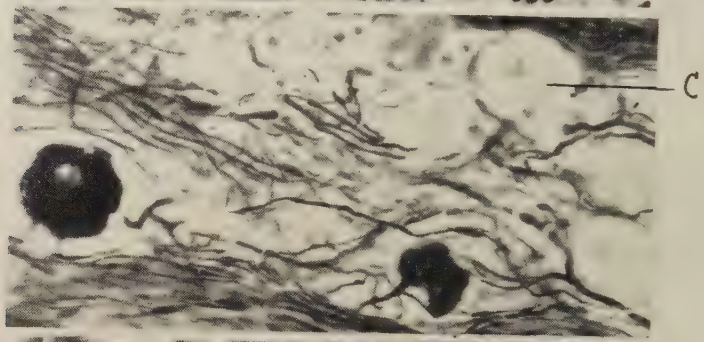

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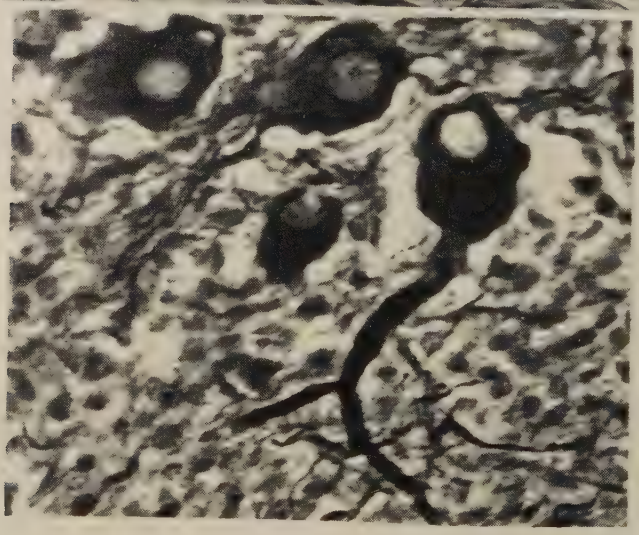

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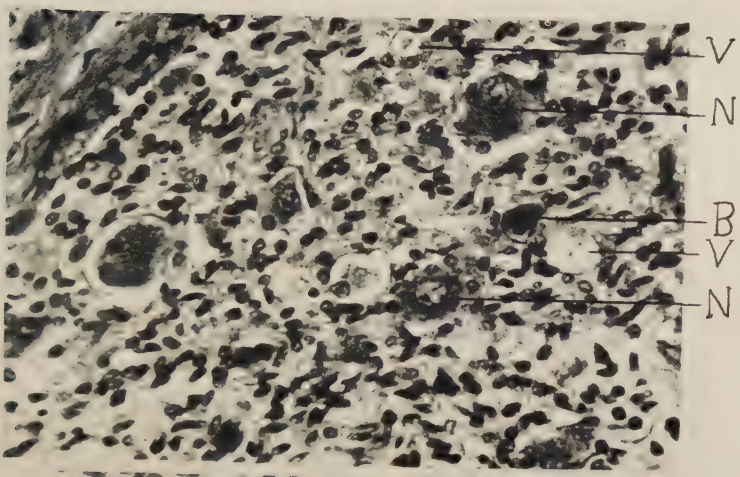

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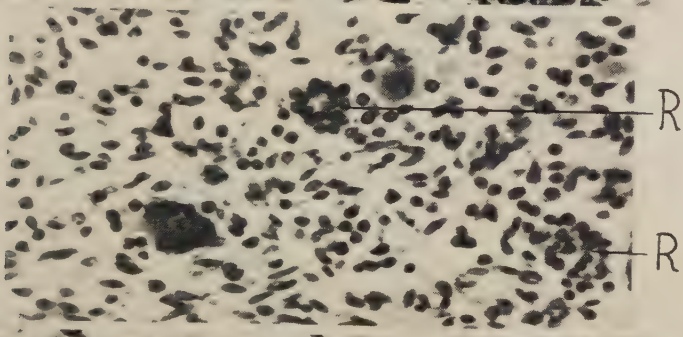

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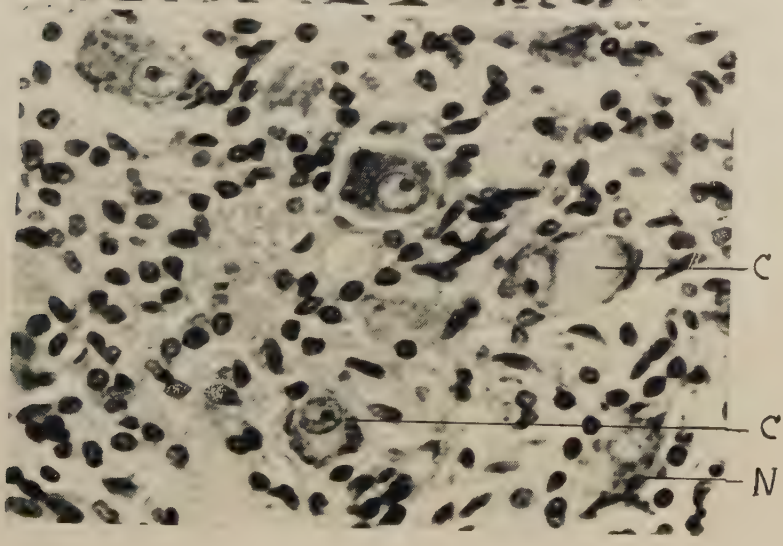

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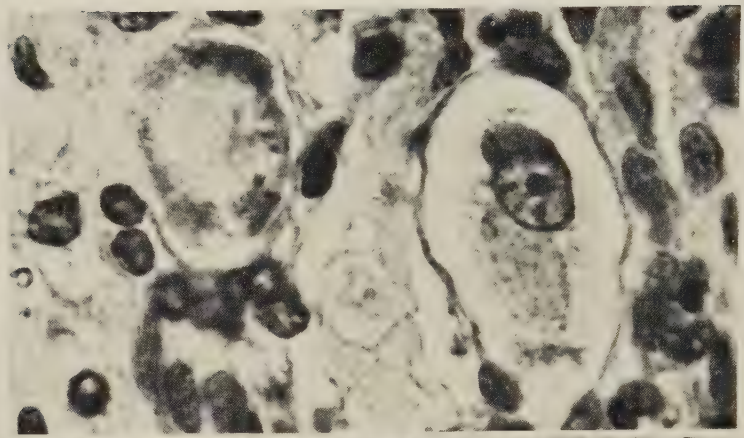

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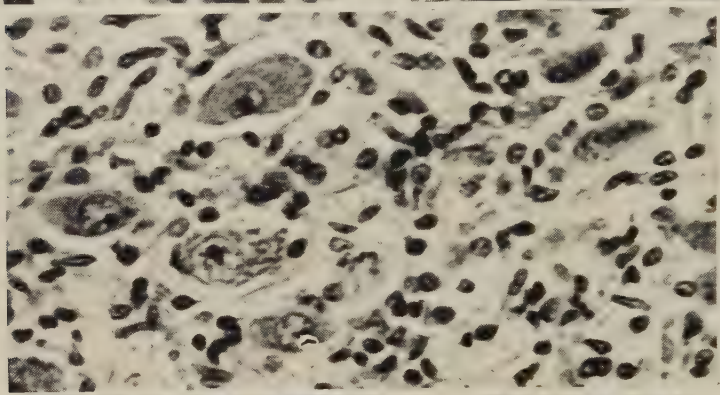

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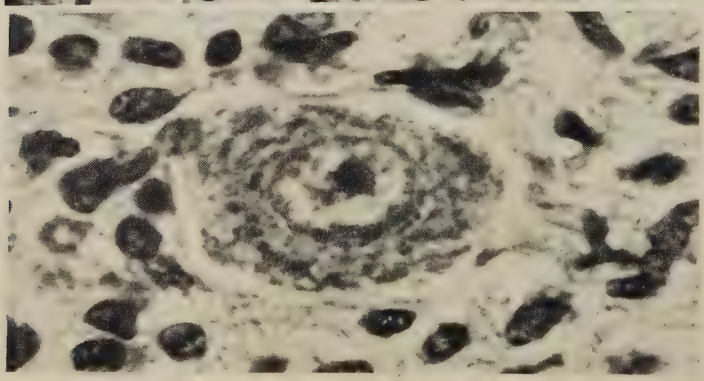

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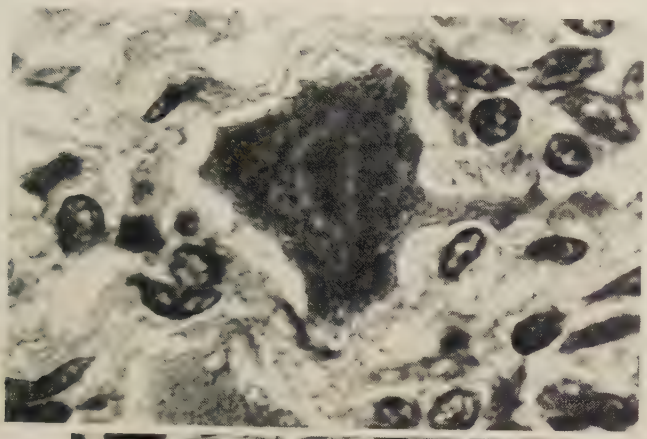

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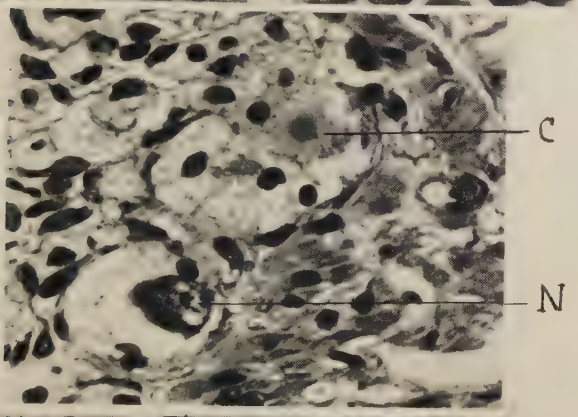

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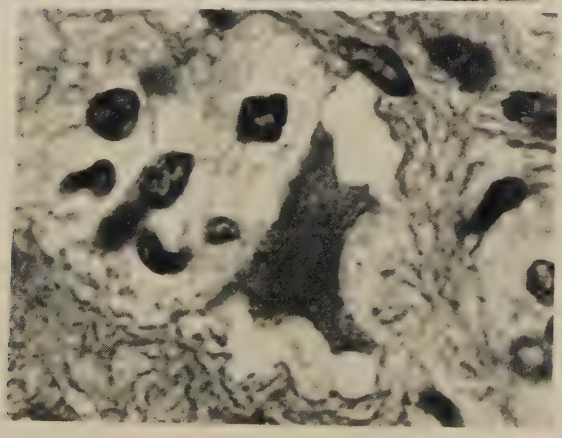

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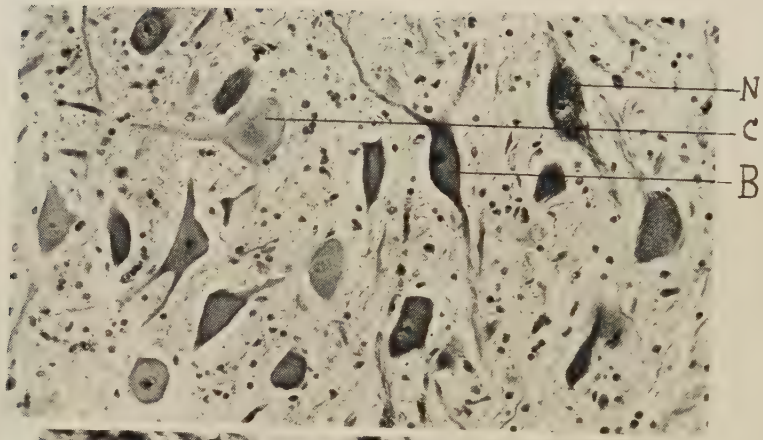

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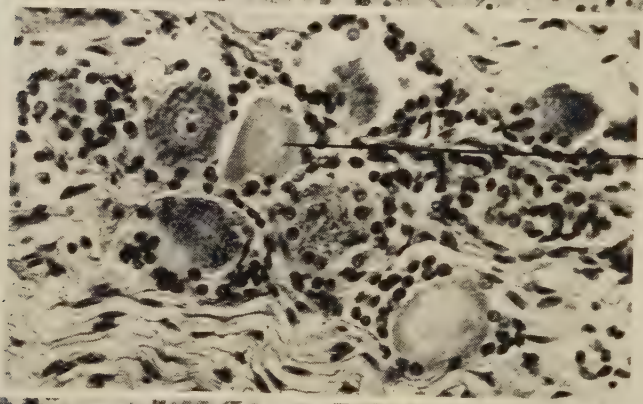

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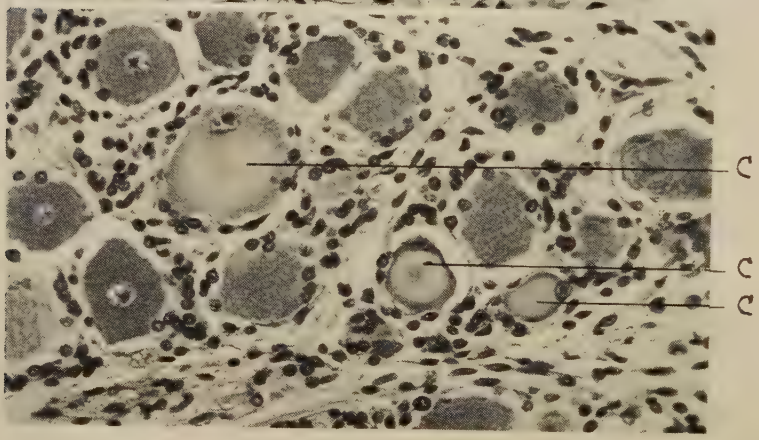

Fig.24

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SOME BIOCHEMICAL AND CLINICAL ASPECTS OF MILK FEVER

By

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INTRODUCTION

During the spring and summer of 1948 a considerable number of cases of milk fever which did not respond typically to calcium therapy were encountered in the clinical practice of the Royal (Dick) Veterinary College. This had been especially the case on one particular farm where, in the course of a few weeks, four cows had been lost following failure to respond to calcium administration. On this farm resort to udder inflation therapy had proved successful where calcium therapy had failed. An account of these and other cases has been published by Robertson, Burgess, Marr and Milne, (1948). Garm (1950) has reported that in recent years in Norway and Sweden calcium therapy and udder inflation have been far less effective in the treatment of milk fever. McBarron (1952) in Australia has observed an increased number of relapses following treatment and states that these cases constitute a difficulty in further treatment.

Blood examination in the cases reported by Robertson, *et al.* suggested that the beneficial results of udder inflation might be associated with its ability to raise the plasma inorganic phosphate as well as the serum calcium. Barker (1948) has suggested that hypophosphataemia is a complicating factor in atypical cases of milk fever. He claimed good results from the parenteral administration of a solution incorporating one ounce of acid sodium phosphate with the calcium, supplemented by the oral administration of the same salt for two days.

There have been very few studies of the biochemical effects of calcium treatment. The present paper gives an account of the effect of calcium therapy and of udder inflation on the serum calcium, serum magnesium, and plasma inorganic, total acid soluble, lipid and total phosphates in cases of milk fever and discusses the relationship between the data obtained and the response of the animals to treatment.

MATERIALS AND METHODS

Animals. The animals involved consisted mainly of cases of milk fever reported for treatment to the Clinical Department, Royal (Dick) School of Veterinary Studies, Edinburgh. The predominant breed was Ayrshire.

Sampling. Blood samples were taken immediately before treatment, usually from the jugular vein. As many samples as possible

* Now at the University of Queensland Veterinary School, Brisbane.

were taken after treatment. In nearly all cases a second sample was taken 15 to 30 minutes after treatment but as the cases were all routine ones encountered in clinical practice it was not always possible to take as frequent blood samples thereafter as was desired. Accordingly, there was considerable variation in the numbers of post-treatment samples taken and in the time relative to treatment at which they were taken.

Methods of treatment. For the purposes of the investigation only two forms of treatment were used, viz. the intravenous injection of four ounces of calcium borogluconate in 400 ml. water, and the inflation of the udder with air, the latter being resorted to only when calcium therapy failed to produce a favourable response.

Blood analysis. Serum calcium was estimated by the Clark and Collip (1925) modification of the Kramer-Tisdall method. After precipitating the calcium the supernatant fluid was used for estimating serum magnesium by the method of Denis (1922) modified according to Hawk (1947). The plasma inorganic phosphate was determined as soon after collection as possible, using the method of Fiske and Subbarow (1925). The organic phosphate estimations were adapted from the method of Greenhill and Pollard (1935) ; the preparation of the extracts and the " blueing-up " were by the method of Fiske and Subbarow (1925). Colorimetric estimations of phosphate and magnesium were carried out in a " Spekker " absorptiometer.

All estimations were carried out in duplicate, and if the results differed by more than 5 per cent. the estimations were repeated.

Statistical analyses were carried out as described by Snedecor (1946).

RESULTS

Data have been collected from 96 cows affected with milk fever. Of these, 67 responded to first treatment, 29 required a second treatment, four a third and one a fourth, making a total of 130 treatments. Of these, 119 were by calcium injection and 11 were by udder inflation.

Levels of Serum Calcium, Serum Magnesium and Plasma Phosphates before Treatment

These are shown in Table 1. A comparison is also made between the levels of these fractions prior to first treatment and prior to second and subsequent treatments. It will be seen that the serum calcium, plasma total acid soluble phosphate and plasma total phosphates were significantly but not markedly increased at the time of the second treatment. Table 1 also shows a comparison between the blood fraction levels in milk fever and those in normal third to sixth calf cows (Moodie, Marr and Robertson, 1955) between 16 and 24 hours after calving which is the time when the majority of our milk fever cases occurred (see below). It will be seen that while the

TABLE 1

BLOOD LEVELS BEFORE TREATMENT IN ALL CASES OF MILK FEVER

		Serum calcium mg. %	*Serum magnesium mg. %*	*Plasma Inorganic phosphate mg. %*	*Plasma total acid soluble phosphate mg. %*	*Plasma lipid phosphate mg. %*	*Plasma total phosphate mg. %*
1. All treatments	No. of Treatments	129	128	130	88	88	88
	Mean	5.01	3.22	1.60	2.09	3.29	5.37
	S.E.	0.119	0.067	0.084	0.121	0.144	0.195
2. **First** treatments	No. of Treatments	96	95	96	59	59	59
	Mean	4.83	3.22	1.54	1.84	3.17	5.07
	S.E.	0.120	0.083	0.099	0.111	0.172	0.210
3. Second or subsequent treatments	No. of Treatments	33	33	34	29	29	29
	Mean	5.52	3.21	1.74	2.60	3.51	6.00
	S.E.	0.293	0.104	0.155	0.269	0.260	0.391
4. Significance of difference between 2 and 3		5%	Not Sig.	Not Sig.	1%	Not Sig.	5%
5. Third-Sixth calving normal cows, 16-24 hours after calving	No. of Treatments	16	16	16	8	7	8
	Mean	8.94	3.25	4.65	6.22	3.21	9.10
	S.E.	0.303	0.152	0.237	0.585	0.593	0.465
6. Significance of difference between 2 and 5		1%	Not Sig.	1%	1%	Not Sig.	1%

serum calcium, plasma inorganic phosphate, plasma total acid soluble phosphate and plasma total phosphate were significantly lower than in normal parturient cows, there were no significant differences in serum magnesium or in plasma lipid phosphate.

Number of Calving at which Milk Fever Occurred

Information on this was available in 93 of the cases. Two cases occurred at the second calving, 21 at the third, 35 at the fourth, 19 at the fifth, nine at the sixth, six at the seventh and one at the eighth. The age distribution of the population from which these cases were drawn is not known, but it is obvious that the incidence increases until at least the fourth calving.

Time Relative to Calving at which Milk Fever Occurred

This was known in 93 cases of which 85 (81.4 per cent) occurred between 10 hours before and 96 hours after calving, the average interval between calving and first treatment for these cases being 16.5 hours. The distribution of the eight remaining cases was 14 days before calving, and five days, six days, seven days, ten days, two weeks, three weeks and four months after calving.

Pre-treatment Blood Levels in Relation to Severity of Clinical Signs

Complete clinical histories of 114 treatments in 85 cows are available. These have been classified into mild, moderate or severe according to the nature of the clinical signs without reference to the blood levels. The general guiding principle was that cases which were still on their feet at the time of treatment were classified as " mild "; cases in sternal recumbency were classified as "moderate"; and cases in lateral recumbency were classified as " severe." The only exceptions to this rule were cases which were in sternal recumbency but which were described in the clinical history as " alert " or " showing mild symptoms "; These were classified as " mild." A comparison has been made of the pre-treatment levels of the blood fractions in the three different clinical categories and is shown in Table 2.

It will be seen that there was a well marked correlation between the severity of the clinical signs and the extent of the depression in serum calcium, plasma inorganic and plasma total acid soluble phosphates, and a less well marked correlation with plasma total phosphate. There was no significant correlation between the severity of the signs and the levels of serum magnesium or plasma lipid phosphate.

Response to Treatment

Classification of cases. Cases have been classified as showing a satisfactory or an unsatisfactory response to treatment. A satisfactory response has been taken as one where the cow appeared normal within 10 hours of treatment and did not subsequently relapse. In the majority of cases classified as showing an unsatisfactory

TABLE 2

PRE-TREATMENT BLOOD LEVELS IN RELATION TO CLINICAL HISTORY

Clinical signs		*Serum calcium* mg. %	*Serum magnesium* mg. %	*Plasma inorganic phosphate* mg. %	*Plasma total acid soluble phosphate* mg. %	*Plasma lipid phosphate* mg. %	*Plasma total phosphate* mg. %
Mild	No. of treatments	44	45	45	35	35	35
	Mean	5.90	3.20	2.00	2.58	3.25	5.87
	S.E.	0.217	0.100	0.152	0.229	0.267	0.347
Moderate	No. of treatments	55	54	55	38	38	38
	Mean	4.57	3.27	1.38	1.90	3.36	5.23
	S.E.	0.138	0.112	0.092	0.135	0.186	0.231
Severe	No. of treatments	14	13	14	9	9	9
	Mean	3.85	3.37	1.01	1.37	3.08	4.40
	S.E.	0.207	0.258	0.172	0.175	0.361	0.480
	F	xx	N.S.	xx	xx	N.S.	x
		Mild>> Mod.> Sev.		Mild>>Mod.	Mild>>Mod.		Mild> Sev.
		Mild>>Sev.		Mild>>Sev.	Mild>>Sev.		

>> Significantly greater at 1%

> Significantly greater at 5%

response there was a definite relapse, with recurrence of clinical signs after first treatment. In a few cases classified as unsatisfactory the cows did not show complete recurrence of the clinical signs of milk fever but merely dullness and anorexia and an improvement following further treatment. It was possible to classify on this basis 68 cases of milk fever of which 40 (59 per cent) responded satisfactorily, i.e. they required only one treatment. Of the 28 cows

FIGURE 1. RELATION BETWEEN TIME FROM CALVING AND RESPONSE TO FIRST TREATMENT IN MILK FEVER CASES

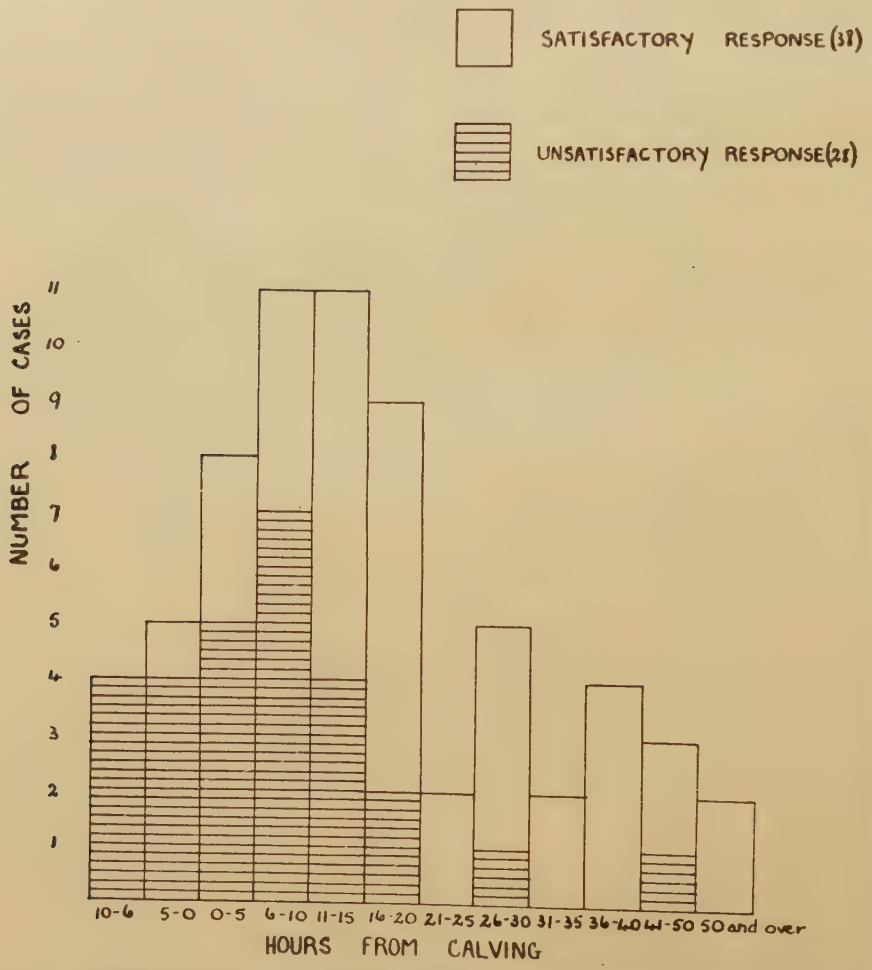

Fig. 1. Relation between time from calving and response to first treatment in Milk Fever cases.

showing an unsatisfactory response, 23 responded to a second treatment, three responded to a third treatment and one responded after four treatments. One cow died without responding to two treatments, biochemical examination showing that just prior to death the blood fractions were within the normal range ; post mortem examination failed to reveal any definite cause of death.

Time from calving in relation to response to treatment. The time between calving and first treatment was known for 66 of the 68 cases classified according to response to treatment. The average interval between calving and first treatment for the 66 cows was 16 hours. Thirty-eight cows showing a satisfactory response were treated at an average time of 23 hours after calving ; the 28 cows showing an unsatisfactory response were treated at an average time of 6.5 hours after calving—the difference between the groups being significant at 1 per cent (Fig. 1). Eight of the nine cases occurring before calving showed an unsatisfactory response to first treatment.

Initial blood levels in relation to response to treatment. Comparisons were made between the initial blood levels in all treatments which resulted in a satisfactory response and those in all treatments which resulted in an unsatisfactory response. This showed that, in this respect, there were no significant differences between the groups.

Severity of clinical signs in relation to response to treatment. The correlation between the nature of the clinical signs and the nature of the response to treatment was as follows :—

Clinical Signs	*Satisfactory response*	*Unsatisfactory response*
Mild	21	9
Moderate	21	22
Severe	4	2

The Chi-square test failed to reveal any statistically significant differences in response according to the nature of the clinical signs although there was a tendency for cases showing moderate clinical signs to be more difficult to treat than those with mild or severe symptoms. This is in keeping with general field experience.

Blood levels after treatment with calcium borogluconate in relation to response to treatment. After the intravenous injection of four ounces of calcium borogluconate there was an increase in serum calcium which varied from 6 to 10 mg. per cent. at 15 minutes after the injection (Fig. 2). This was followed by a progressive decline in level until 10 hours after treatment when the levels approximated the pretreatment values. In cases where there was no relapse this fall in serum calcium was succeeded by a progressive rise over two or three days until normal levels were re-established (Fig. 3). In the period between treatment and 10 hours after treatment, however,

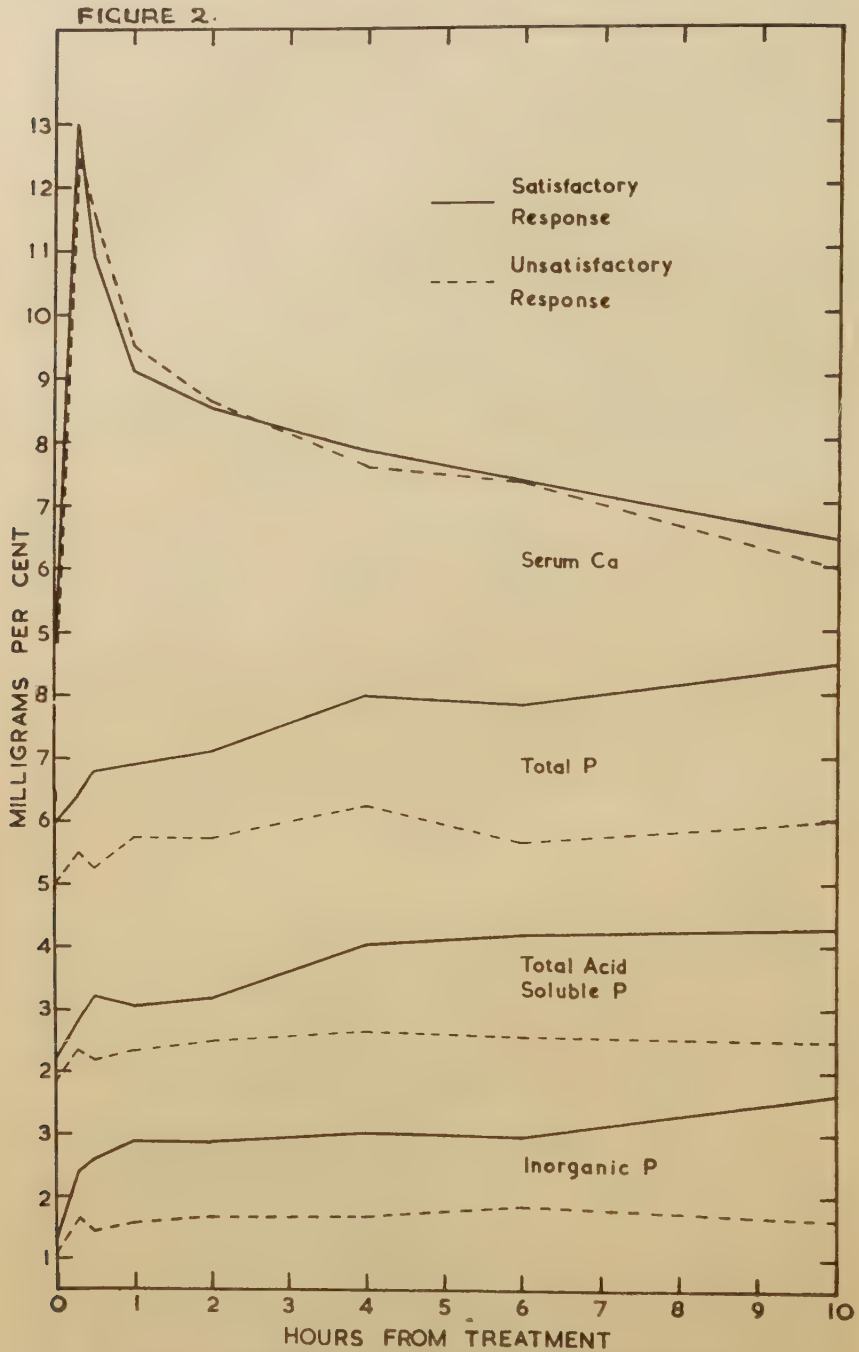

Fig. 2. The effect of calcium therapy on serum calcium and plasma phosphate levels in cases showing a satisfactory and in cases showing an unsatisfactory response to treatment.

the behaviour of serum calcium was more or less the same in the satisfactory and unsatisfactory response groups.

The plasma phosphates also increased after calcium therapy. Fig. 2 shows that the rise in plasma phosphate in the satisfactory response group was rapid and progressive until 10 hours after treatment. In the unsatisfactory group the rise in phosphate immediately after injection was less well marked and thereafter the levels remained fairly constant. The significance of these changes is tested in Tables 3 and 4. Table 3 shows a comparison between the blood constituents before treatment and up to 10 hours after treatment in five cows which responded satisfactorily and five which responded unsatis-

TABLE 3

BLOOD FRACTION LEVELS BEFORE AND AFTER TREATMENT WITH CALCIUM BORO GLUCONATE IN COWS GIVING SATISFACTORY AND UNSATISFACTORY RESPONSES

(Means of five cows)

Time from treatment	*Serum calcium* Mg. %		*Plasma inorg. phosphate* Mg. %		*Plasma total acid soluble phosphate* Mg. %		*Plasma total phosphate* Mg. %	
	S.	*U.*	*S.*	*U.*	*S.*	*U.*	*S.*	*U.*
Before treatment	3.49	4.02	1.22	1.10	1.45	1.71	5.08	5.18
After treatment								
15 mins.	9.76	14.59	2.09	1.54	2.35	2.33	5.81	5.70
30 mins.	9.10	11.63	2.15	1.54	2.40	2.54	6.02	5.98
1 hour	8.15	10.44	2.24	2.16	2.42	2.90	6.18	6.40
2 hours	7.30	8.91	2.28	2.25	2.66	2.96	6.48	6.66
4 hours	6.41	7.44	2.38	1.85	3.18	2.79	6.55	6.04
6 hours	6.16	6.94	2.43	1.79	3.13	2.95	6.48	6.04
10 hours	5.84	5.74	3.59	1.72	3.98	2.63	7.72	6.64
Mean	7.03	8.71	2.30	1.74	2.68	2.59	6.29	6.08
S.E.	0.679	0.630	0.229	0.198	0.286	0.255	0.302	0.276
Sig.	1%	1%	1%	1%	1%	5%*	1%	5%

S = Satisfactory response U = Unsatisfactory response

*Since there is one missing plot in each series it would be wise to consider this value as Not Significant (Snedecor, G.W. Statistical Methods. 4th ed. 1946. The Iowa State College Press. p. 269).

factorily to treatment. It will be seen that there are significant changes in all fractions with the possible exception of plasma total acid soluble phosphate in the unsatisfactory response group. There is little difference between the groups in the nature of the changes in serum calcium. With the plasma phosphates, however, it is evident that the satisfactory group showed a steady and progressive increase throughout the period of observation, whereas in the unsatisfactory group the phosphates reached a peak at about two hours and either declined or remained relatively steady thereafter.

Table 4 is based on a larger number of treatments where three samples were taken, one before treatment, one 15 to 30 minutes after

treatment and a third sample between 2 and 10 hours after treatment. Increases in level from treatment to 15 to 30 minutes after treatment and to the third sample have been calculated for both response groups and the increases compared by Student's t test. It will be seen that the initial increases in the blood constituents, i.e. at 15 to 30 minutes, do not differ significantly except in the case of total acid soluble phosphate, but that at the third sample the phosphates of the satisfactory group were significantly higher than the unsatisfactory group. The serum calcium had increased by the same amount in both groups.

TABLE 4

INCREASES IN BLOOD FRACTIONS OF MILK FEVER CASES AFTER TREATMENT WITH CALCIUM BOROGLUCONATE

Fraction		*Increase at* Second sample (*15-30 Minutes*)		Third sample (*2-10 Hours*)	
		Response S.	U.	S.	U.
	No. of cases	*25*	*15*	*17*	*10*
Serum Calcium	Mean Mg. %	8.15	6.37	3.15	3.22
	S.E.	0.512	0.754	0.463	0.793
	Difference	Not Sig.		Not Sig.	
Plasma Inorganic P.	Mean Mg. %	0.75	0.69	1.73	0.81
	S.E.	0.113	0.133	0.186	0.239
	Difference	Not Sig.		Sig. at 1%	
	No. of cases	23	*15*	*17*	*10*
Plasma Total acid Soluble P.	Mean Mg. %	1.10	0.55	2.11	0.97
	S.E.	0.146	0.156	0.188	0.220
	Difference	Sig. at 5%		Sig. at 0.05%	
Plasma Lipid P.	Mean Mg. %	−0.01	0.00	0.04	0.12
	S.E.	0.108	0.118	0.135	0.1117
	Difference	Not Sig.		Not Sig.	
Plasma Total P.	Mean Mg. %	0.97	0.53	2.16	1.14
	S.E.	0.175	0.175	0.235	0.251
	Difference	Not Sig.		Sig. at 1%	

S. = Satisfactory U. = Unsatisfactory

Treatment by Udder Inflation

Results of treatment by udder inflation are shown in Table 5 for five cases for which sufficient samples were available to warrant analysis. This shows that there were significant increases in serum calcium and magnesium and in the plasma phosphates except the lipid fraction. The increases were gradual over a period of about two hours and thereafter were slight.

TABLE 5

BLOOD FRACTION LEVELS BEFORE AND AFTER TREATMENT BY UDDER INFLATION

Time for treatment	*Serum calcium*	*Serum magnesium*	*Plasma inorganic phosphate*	*Plasma total acid soluble phosphate*	*Plasma total phosphate*
	Mg. %	*Mg. %*	*Mg. %*	*Mg. %*	*Mg. %*
Before treatment	5·31	2.74	0.74	1.26	3.26
After treatment					
15 mins.	6.12	2.87	1.33	1.63	4.08
30 mins.	6.71	3.09	1.80	2.28	5.02
1 hour	7·35	3.18	1.83	3.36	5.90
2 hours	7·71	3.30	2.99	3.96	6.34
4 hours	7·74	3.29	3.90	4.32	6.86
6 hours	7.81	3.18	2.84	4.04	6.84
10 hours	7·35	3.34	4.03	4.55	7.26
No. of cows	5	4	5	5	5
Mean	7.01	3.12	2.43	3.18	5.70
S.E.	0.327	0.085	0.0455	0.372	0.343
Sig.	1%	1%	1%	1%	1%

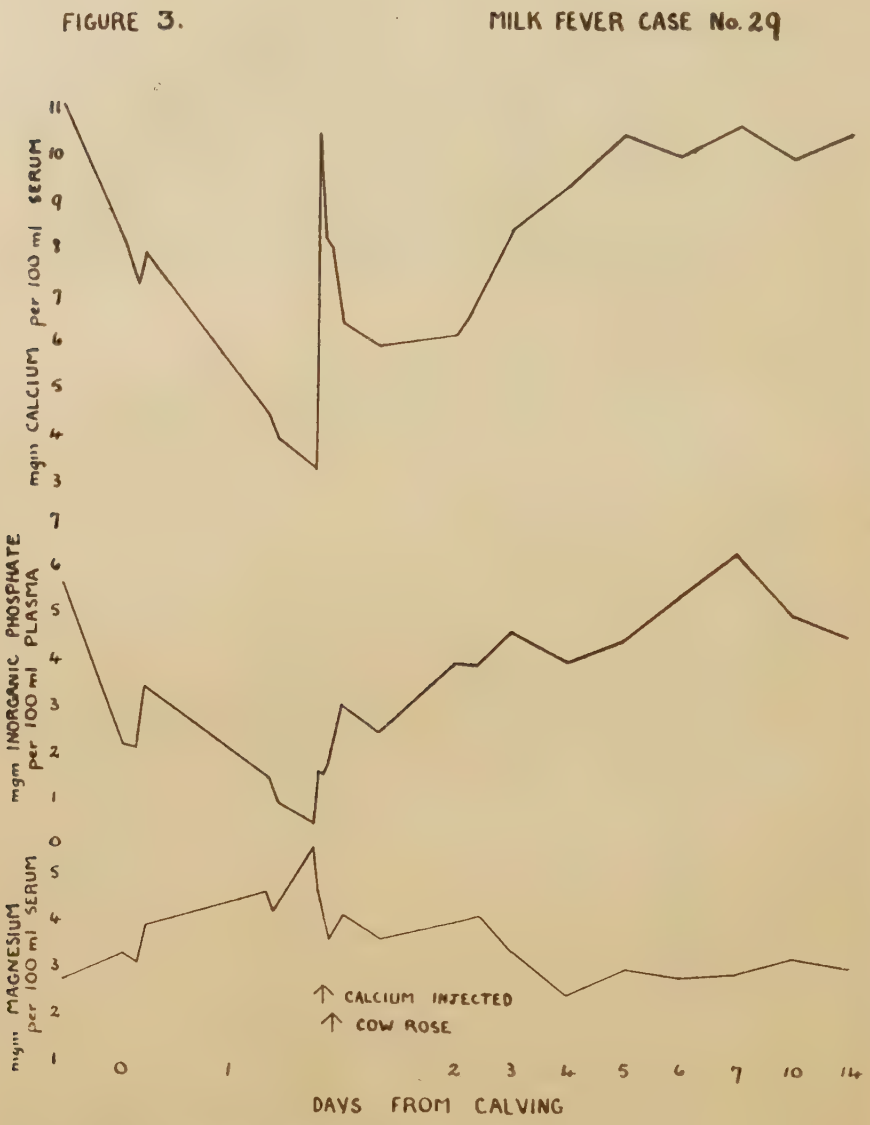
FIGURE 3.
MILK FEVER CASE No. 29
mgm CALCIUM per 100 ml SERUM
mgm INORGANIC PHOSPHATE per 100 ml PLASMA
mgm MAGNESIUM per 100 ml SERUM
CALCIUM INJECTED
COW ROSE
DAYS FROM CALVING

Fig. 3. Typical satisfactory response to calcium therapy.

Detailed Studies of Response to Treatment

In fourteen cases of milk fever the post-treatment blood levels were studied in considerable detail. Most of the data from these cases have been abstracted and used in the preparation of the above results. However, the complete data are valuable in showing the trends in the behaviour of the blood fractions following treatment. The concise and orderly presentation of the complete data from these cases in tabular form is rendered impossible by the fact that while

FIGURE 4. MILK FEVER CASE No. 31

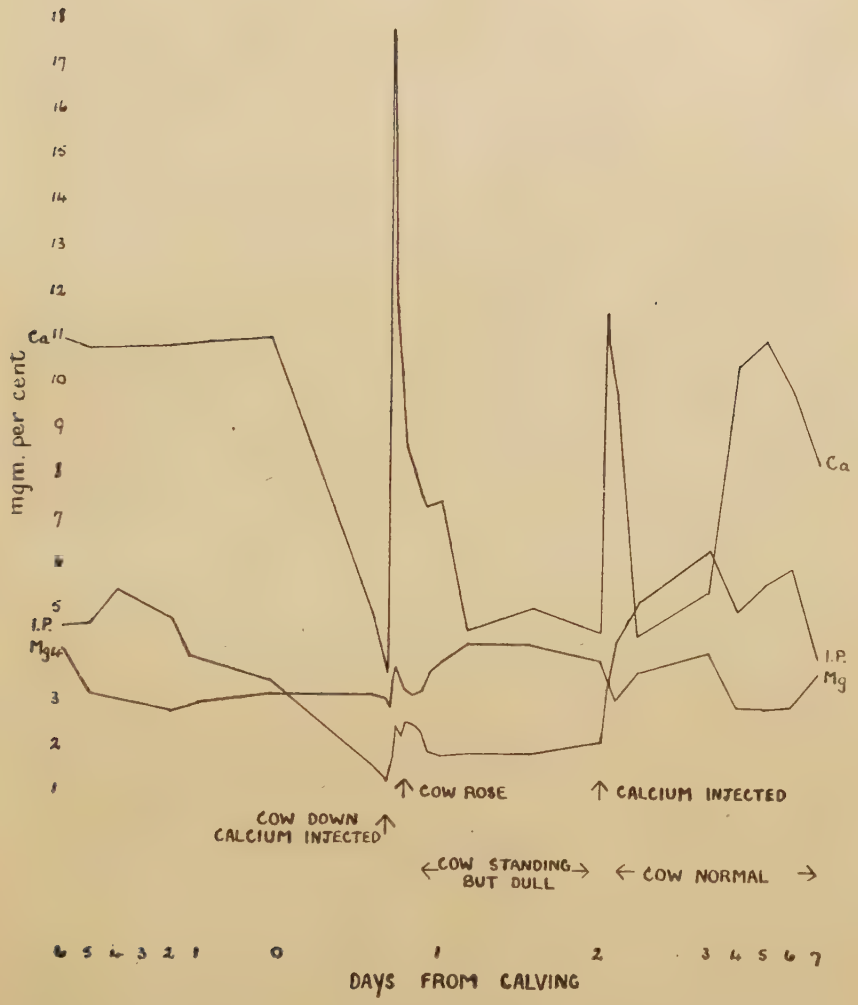

Fig. 4. Unsatisfactory initial response to calcium therapy.

some cows required only one treatment for recovery, others required two, three and in one case four treatments before eventual recovery. This made it impossible to take the post-treatment samples at exactly the same intervals. Accordingly, graphs have been prepared from the data of some of these animals, showing different types of case encountered and are used to illustrate points in the discussion of results. (Figs. 3 to 5).

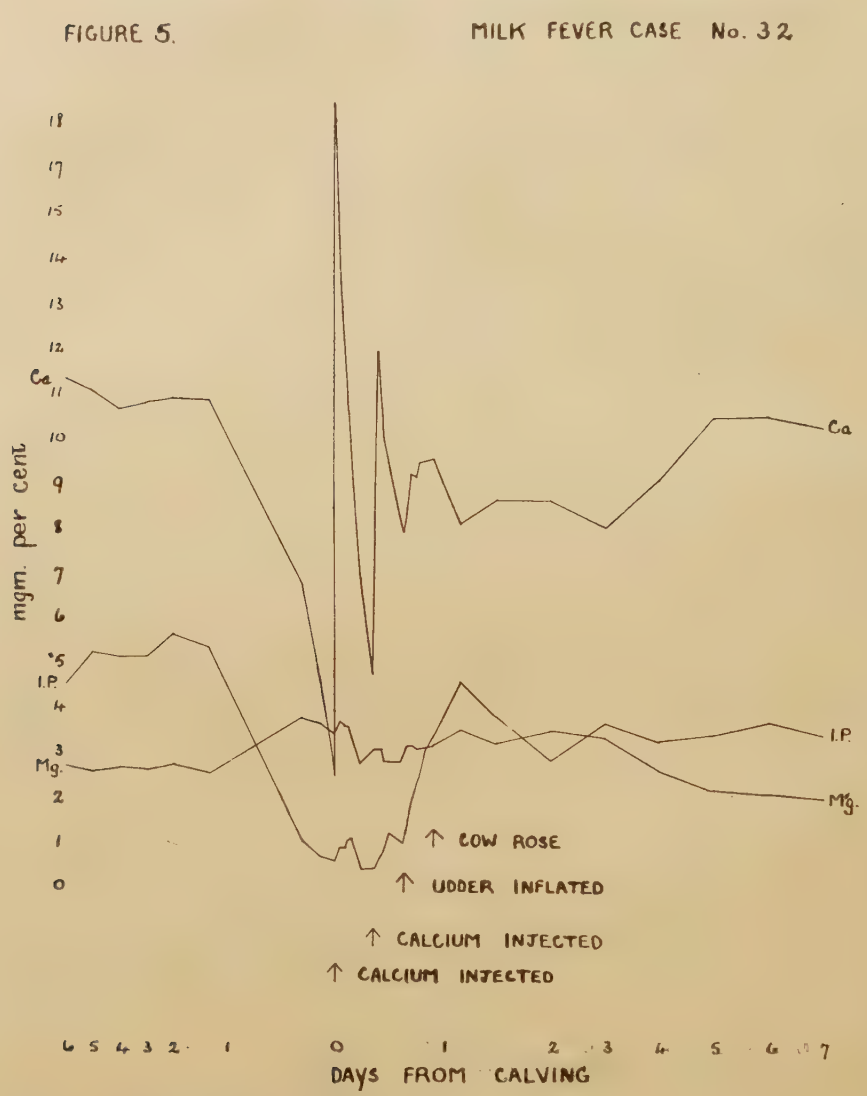

Fig. 5. Failure to respond to calcium therapy.

DISCUSSION

The pre-treatment levels of serum calcium, serum magnesium and plasma inorganic phosphate in milk fever cases in this investigation agree fairly closely with those of previous workers. The results confirm the already well established fact that milk fever is usually associated with hypocalcaemia, hypophosphataemia and hypermagnesaemia. The hypocalcaemia and hypophosphataemia were more marked than in normal parturient cows of similar ages. In the

cases investigated here, however, the level of serum magnesium was slightly lower than it was in normal parturient cows of an age susceptible to milk fever. The fact that the level of serum magnesium was no higher in milk fever than in normal parturient cows and that there was no significant correlation between the level of serum magnesium and the severity of the clinical signs suggests that serum magnesium does not play a very important role in typical milk fever. Hibbs (1950) has suggested that it is possible that the relatively high serum magnesium level accounts for the lack of tetanic symptoms in typical milk fever and for the comatose condition which usually is seen in spite of the low blood calcium, but the levels attained in our cases were hardly high enough to support such a view.

Barker (1939) has classified hypocalcaemia into three clinical groups according to whether it is accompanied by a raised, lowered or normal level of serum magnesium. He claims that hypocalcaemia and hypermagnesaemia gives a clinical picture of " reeling, paresis and narcosis " ; cases with hypocalcaemia and a normal level of serum magnesium show " paddling, paresis and coma " ; and cases with hypocalcaemia and hypomagnesaemia show " excitement and convulsions." It is possible to agree with the last of Barker's groups, i.e. hypocalcaemia and hypomagnesaemia, which is essentially the blood picture in grass tetany, where the clinical signs are usually recognisably different from those in true milk fever. In this investigation no cases of milk fever with marked hypomagnesaemia were encountered—the lowest level of serum magnesium observed was 1.40 mg. per cent. in a cow which developed milk fever four days after calving. It was less easy to classify cases into the other clinical groups suggested by Barker but in the present study, the group most nearly approaching Barker's " comatose " type (the group showing severe symptoms of milk fever, Table 2), showed a slightly higher mean level of serum magnesium than the group most nearly approaching Barker's " narcotic " type viz. the group showing moderately severe symptoms.

The finding that the severity of the clinical signs was significantly related to the extent of the depression in serum calcium agrees with the original observations of Little and Wright (1925) and with those of Dryerre and Greig (1928) and is in contrast to the findings of Blood, Larsen and White (1949) that there was no correlation between the severity of the clinical signs and the extent of the depression in serum calcium in a study of 25 cases of milk fever. In the present study there was also a significant correlation between the severity of the clinical signs and the extent of the depression in plasma inorganic and total acid soluble phosphates and a less marked correlation in the case of plasma total phosphate. There was no correlation in the case of plasma lipid phosphate.

That the clinical picture is not solely dependent on the level of serum calcium, however, is evident from a study of the levels after

treatment with intravenous calcium borogluconate (Fig. 2). This shows that in the group of cases which responded satisfactorily to calcium treatment the serum calcium had returned almost to its pre-injection level by 10 hours after treatment although none of the cows were exhibiting clinical signs of milk fever at this time. The finding that a distinct hypocalcaemia frequently recurs even in cases which do not relapse following treatment by intravenous calcium injection does not agree with the views of Hibbs, Krauss, Pounden, Munroe and Sutton, (1946) and Hibbs, Pounden and Krauss, (1951). These workers state that the decline in serum calcium following treatment levels off somewhere near the normal level, and that in cases which relapse, the fall in serum calcium continues until milk fever levels are again reached. In this study it was found that even in cases which did not relapse the fall in serum calcium continued until levels which were little higher than the pre-treatment values were reached and then a slow rise in serum calcium was instituted with a return to normal levels in three to four days. In Fig. 3 it will be noticed that the blood calcium fell to below 6 mg. per cent. even though the animal remained normal and in Fig. 4 following the second injection of calcium salts the animal appeared perfectly healthy although blood analysis revealed a serum calcium level well below 5 mg. per cent.

The behaviour of serum calcium following treatment by intravenous calcium borogluconate injections suggests that in successful cases calcium injection, by inducing a marked though temporary rise in the blood calcium level, abolishes the clinical signs for long enough to allow the calcium regulating mechanisms time to re-establish normal levels. In other words calcium injection acts as a temporary expedient until the normal regulatory mechanisms come into play rather than by any direct specific curative action. The observations of Niedermeier, Smith and Whitehair (1949) on mastectomised cows, and of Robertson, Marr and Moodie (1955) on mastectomised and pre-partum milked cows suggest that the main cause of the fall in serum calcium is the drain produced by the secretion of colostrum. It follows, therefore, that calcium injection therapy may serve only to counteract the effects of the drain of mammary secretion until the calcium regulating mechanisms have time to restore normal levels. Hibbs *et al.* (1951) have arrived at somewhat similar conclusions.

It has been found that milk fever cases occurring before calving, or within a few hours after calving, are more likely to relapse following treatment than are cases occurring later in relation to calving. This suggests that in these animals it usually requires some two to three days for the normal calcium regulating mechanisms to compensate for the demands for calcium produced by the sudden increase in secretion of colostrum at parturition. This is in keeping with our observations on normal parturient cows (Moodie, Marr and Robertson, 1955) and suggests that the sequence of events is essenti-

ally similar in the two cases. Thus in a case given intravenous calcium therapy either before or shortly after calving it is likely that the effect of the artificial raising of the blood calcium will not last sufficiently long for the normal regulating mechanisms to come into play and restore normal levels. Conversely, in a case occurring, say 24 hours after calving the effect of the raised blood calcium level is maintained sufficiently long for the calcium regulating mechanisms to restore normal levels. It follows, therefore, that anything which will serve to maintain the serum calcium level will lessen the likelihood of relapse. The rapid decline in serum calcium level and the high percentage of relapses following intravenous calcium therapy observed in this study confirm the advantage of supplementing intravenous therapy by subcutaneous injections.

The results confirm the already well established fact that milk fever is accompanied by a low level of plasma inorganic phosphate, and demonstrate that there is also a depression of plasma total acid soluble and plasma total phosphates. Plasma lipid phosphate appears to be little altered. There have been only limited studies of the effect of calcium therapy on the plasma phosphate levels in milk fever (Robertson *et al.*, 1948 ; Robertson, 1949). Our results show that calcium injection therapy is followed by an immediate rise in the levels of the plasma phosphates. This rise is most marked in the case of plasma inorganic phosphate which may increase by 100 per cent. within 30 minutes of injection (Fig. 3).

That a rise in the level of the plasma phosphates is necessary for satisfactory recovery is suggested by the results obtained in a comparison of satisfactory and unsatisfactory responses to calcium therapy. In Fig. 2 it will be noted that although the behaviour of serum calcium was similar in the two groups, in the satisfactory response group there was a sustained rise in the level of the phosphate fractions, whereas in the unsatisfactory response group the plasma phosphates showed only a slight initial rise and then declined or remained level. This is also suggested by the findings illustrated in the individual cases (Figs. 3 to 5), where successful treatment was accompanied in every case by a marked rise in plasma inorganic phosphate, whereas following unsuccessful treatments this fraction usually rose much less markedly and frequently relapsed. It would seem, therefore, that the response of the plasma phosphates to calcium therapy may be of some importance in determining the nature of the clinical response to treatment. The results obtained from udder inflation (Table 5) in this study confirm the finding of Robertson (1949) that it causes a marked consistent rise in the level of blood phosphates. This is well illustrated also in Fig. 5.

These results could be interpreted as supporting the suggestions of Fish (1930) and of Barker (1948) that phosphate as well as calcium should be used in milk fever therapy. It does not follow, however, that the artificial raising of the blood phosphate level by the injection of phosphate will have any beneficial clinical effect, particularly in

view of the fact that intravenous injection of acid sodium phosphate can cause some depression in blood calcium level (Robertson, Marr and Stewart—unpublished observations).

The factors causing the fall in plasma phosphates in normal parturient cows and in milk fever are not fully understood. Two observations, however, suggest that the act of parturition has more influence on the plasma phosphate levels than the onset of milk secretion, although with serum calcium the opposite appears to be the case. First, in normal parturient cows the plasma phosphate level falls from 12 to 24 hours before calving to reach a minimum level at parturition ; and parturition is followed by an immediate rise in the phosphate levels while serum calcium continues to fall to reach its lowest point on an average 24 hours after calving (Moodie, Marr and Robertson, 1955). Secondly, the changes in plasma phosphates still persist in mastectomised cows at parturition, whereas the fall in serum calcium is abolished (Robertson, Marr and Moodie, 1955). It seems, therefore, that there are fundamental differences in the factors which produce the changes in the two fractions in both normal parturient and milk fever cows. Until more is known concerning the factors influencing the plasma phosphates it is not possible to arrive at a definite opinion on the significance of their role in milk fever.

CONCLUSIONS

A study has been made of the levels of serum calcium, serum magnesium and plasma inorganic, total acid soluble, lipid and total phosphates in clinical cases of milk fever and of the effect of calcium therapy and udder inflation on these blood constituents. The data have been analysed according to the nature of the response to treatment.

Serum calcium, plasma inorganic, total acid soluble and total phosphates were significantly lower than in normal parturient cows of an age susceptible to milk fever. Serum magnesium and plasma lipid phosphate were not significantly altered.

The time relative to calving and the number of the calving at which milk fever occurred is presented for 93 cases of milk fever.

There was a significant correlation between the severity of the clinical signs and the extent of the depression in serum calcium, plasma inorganic, total acid soluble and total phosphates. There was no correlation in the case of serum magnesium and plasma lipid phosphate.

Cases were classified into satisfactory and unsatisfactory response to treatment. Cases in the satisfactory response group occurred at an average time of 23 hours after calving and cases in the unsatisfactory response group at an average time of $6\frac{1}{2}$ hours after calving, the difference being highly significant.

There was no correlation between the initial blood levels of the

various constituents, or the nature of the clinical signs and the nature of the response to treatment.

Comparisons between the two groups in the period between treatment with intravenous calcium borogluconate and 10 hours after treatment showed that the behaviour of serum calcium was similar in the two groups. The behaviour of the plasma phosphates differed in the two groups. Cases showing a satisfactory response showed a sustained rise in the plasma phosphates while cases showing an unsatisfactory response showed only a slight rise in plasma phosphates immediately after injection: they then either remained steady or declined until the end of the 10 hour period.

Udder inflation resulted in a sustained rise in all the blood constituents.

The significance of these findings is discussed.

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PATHOLOGICAL AND PHARMACOLOGICAL EXAMINATION OF A BOVINE TUMOUR OF THE ADRENAL MEDULLA

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INTRODUCTION

Paragangliomata of the bovine adrenal medulla are comparatively rare. Descriptions of such tumours have been given by Susman, McGaughey and Torrance (1929), Jackson (1936), and Montroni (1950), and the veterinary literature on the subject has been summarised by Courteau (1935) and Tamaschke (1951/52). In none of these tumours, however, has a chemical analysis of the active materials been made. The purpose of this paper is to describe a tumour of the bovine adrenal medulla on which a detailed pharmacological examination has been carried out.

MATERIALS AND METHODS

The left adrenal of a 2 to 3 year old graded black bullock weighed 83.0 g. and was obtained 48 hours after slaughter. Segments (1 cm. square and 0.25 to 0.5 cm. thick) were treated as follows :— (1) fixation in 10 per cent formol saline ; by frozen sections stained with Sudan IV, or paraffin sections stained with Anderson's haematoxylin and eosin ; (2) Du Bosque fixative (picric acid-alcohol-formol) ; paraffin sections stained either with haematoxylin and eosin or by the methenamine-silver (hexamine) method of Gomori (1952) ; (3) 3 per cent potassium dichromate for 3 days ; paraffin sections taken to distilled water and then replaced in 3 per cent potassium dichromate for two days before being dehydrated, cleared and mounted ; azure stains were also used, as described by Lillie (1948).

For the pharmacological work the cortex was removed as far as possible by dissection and 53.3 g. of the medulla and tumour were placed in 50 ml. 0.01 N-HCl. This was thoroughly minced, the resulting material centrifuged and 5 ml. of the supernatant precipitated with 4 vol. of ethanol. After further centrifuging, an aliquot of the supernatant was evaporated to dryness at 45 to 50°C. and the residue taken up in a small known volume of 0.01 N-HCl. The clear fluid was assayed for its adrenaline and noradrenaline content by paper chromatography and biological assay (Shepherd and West, 1951). For paper chromatographic estimation of other amines and amino-acids, five ml. of the original supernatant were treated with 4 vol. of a mixture of equal volumes of ethanol and acetone to pre-

cipitate protein and reduce the inorganic salt concentration. After centrifugation and evaporation, the final solution was made so that a 20-fold concentration was achieved. After development of the chromatograms, the paper was sprayed with aqueous potassium iodate, aqueous potassium ferricyanide, a mixture of potassium dichromate and formaldehyde (to produce the fluorescence reaction), ninhydrin in butanol, *p*-nitraniline reagent, Folin and Ciocalteu reagent, or acid and alkaline Pauly reagent. In this way, each of the possible precursors of adrenaline and noradrenaline can be detected (West, 1953).

Solutions of (—) adrenaline and (—) noradrenaline bitartrate in 0.01 N-HCl were used as standards. For the paper chromatographic work, solutions of the following substances were also prepared in 0.01 N-HCl :— hydroxytyramine hydrochloride, (±) dihydroxyphenylalanine (DOPA), (±) dihydroxyphenylserine (DOPS), lactyl-adrenaline (LA), lactyl-noradrenaline (LN), (±) norsynephrine, (±) metanorsynephrine, tyramine hydrochloride, tyrosine and phenylalanine. Most of these drugs were obtained through the courtesy of Sterling-Winthrop Research Institute, New York, Hoffman-la-Roche, Basle, and Bayer Products Ltd., London.

RESULTS AND COMMENTS

Macroscopic Examination

The specimen consisted of the left adrenal surrounded by a normal capsule and adipose tissue. The caudal pole was greatly enlarged, and on examination of the cut surface (Fig. 1), a spherical tumour, 5 cm. x 4 cm. x 4 cm., was seen lying in the medulla of this pole. The brown cortex of the normal cranial pole continued round the whole organ. The yellow medulla of the cranial pole, however, was apparently obliterated on one side of the caudal pole. The encapsulated tumour was mottled, red and pink in colour, and soft in consistency. Several large cysts (up to 2 cm. diameter) containing serosanguineous fluid were present in the substance of the tumour.

Microscopic Examination

The thick capsule showed no abnormality. The cortex was of the normal pattern with occasional stellate melanophores lying between the cortical cells, the latter containing a moderate amount of Sudanophilic material.

The medulla of the cranial pole was composed of irregular cords of ovoid, slightly columnar cells, separated by blood sinuses. The granular cytoplasm of these cells stained purple with haematoxylin and eosin (especially in formol-fixed tissue) and the nuclei were oval and moderately chromatic. No reaction was seen in frozen sections stained with Sudan IV. There was a distinct separation between cortex and medulla but no fibrosis was present. The medulla of the caudal pole surrounding the tumour was essentially similar in pattern to that described above but the cords of cells tended to lie parallel

with the line of junction with the tumour and the sinuses were flattened.

There was a thin but distinct capsule of collagenous fibrous tissue round the tumour (Fig. 2), except in a few regions where no such capsule was visible. At these sites a transition between medullary and tumour cells could be seen.

The tumour was composed of slightly thicker cell cords and cell masses separated from each other by blood sinuses which tended to be larger than those in the normal medulla and which in a few areas showed haemorrhage. These tumour cells were more closely packed together than those of the normal medulla ; their nuclei were of the same size as the medullary cells but there was less stippling of the chromatin. The outline of the cytoplasm of the cells was indistinct and the purple granulation fainter. Very few mitoses were found in the tumour.

Our inability to demonstrate the specific granular structures containing adrenal medullary catechols as mentioned by Hillarp, Hökfelt and Nilson (1954) was presumably due to the delay between slaughter of the animal and the fixation of the tumour, since we have achieved satisfactory results with normal bovine adrenal medulla fixed within three hours of slaughter and processed as described above. This delay may also have resulted in some loss of catechol amine in the tumour and adrenal medulla.

Pharmacological Examination

The assay on the blood pressure and nictitating membrane of the spinal cat showed that the total catechol amine content of the tissue was 5.3 mg/g. fresh tissue, a figure which agrees well with that found by Shepherd and West (1953) in the normal bovine adrenal medulla. Although agreement was reached on the total catechol amine content, the relative amounts of noradrenaline and adrenaline were widely different. Whereas in the normal medulla noradrenaline constitutes 27 per cent of the total, in this tumour the figure was 94 per cent of the total (Table I). Holton (1951) found a similar alteration when she compared the adrenal glands of bullocks with those of calves, so that as with human adrenal medullary tumours the difference is probably related to the fact that the tumour cells are relatively undifferentiated and may thus be more comparable with embryonic tissue than with adult tissue.

Possible Precursors of Adrenaline and Noradrenaline

Despite the 20-fold concentration in amine content in the tumour extract, only adrenaline and noradrenaline were identified. There appeared to be a larger amount of phenylalanine than is normally found in bovine extracts, but hydroxytyramine, DOPA, DOPS, LA, LN, norsynephrine, *m*-norsynephrine and tyramine were not detected. Shepherd and West (1953) have already reported the absence of hydroxytyramine and DOPA in extracts of suprarenal glands of calves.

TABLE I
CATECHOL AMINES (MG./G) IN BOVINE ADRENAL TISSUE

Tissue	*Total amine*	*Adrenaline*	*Noradrenaline*		*Author*
			Amount	*Percentage of total amines*	
Bullock whole gland	2.00	1.30	0.70	35	Holton (1951)
Calf whole gland	4.20	1.20	3.00	71	Holton (1951)
Bullock whole gland	1.62	1.20	0.42	26	Shepherd and West (1953)
Bullock medulla	5.50	4.00	1.50	27	Shepherd and West (1953)
Bullock medullary tumour	5.31	0.31	5.00	94	Present work

CONCLUSIONS

The pathology of a tumour of the adrenal medulla from a bullock is described. Adrenaline and noradrenaline were found and the composition of the secretion indicated a similarity to that of embryonic tissue, since over 90 per cent of the catechol amine fraction consisted of noradrenaline. Hydroxytyramine and dihydroxyphenylalanine were not detected.

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OBSERVATIONS ON THE BIONOMICS OF THE FREE-LIVING LARVAE OF THE LUNGWORM *DICTYOCAULUS FILARIA**

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INTRODUCTION

The life history of the lungworm *Dictyocaulus filaria* is well known and Daubney (1920) has given a full description of the development and morphology of the free-living larvae.

Our knowledge of the bionomics of these larvae is, however, very restricted, although laboratory experiments have been carried out to determine the effects of different physical factors on them. The effect of desiccation on longevity has been investigated by several workers, (Raillet, 1889; Guberlet, 1919), and Daubney, (1920), who also studied the effect of temperature on the rate of development and made observations on the vertical migration of these larvae on the walls of glass culture vessels. Kauzal (1933), Cameron (1933), Pavlov (1935), Skriabine and Schultz (1936), Orlov, Alikaev, Priselkov, Rodionov, Gureva and Samartsev (1937), Gerichter (1951), and Soliman, (1953), have all recorded the effects of one or more of these factors.

Observations under natural conditions are less numerous. Hobmaier and Hobmaur (1929), noted that in the field, development from the first to the infective stage took six days at certain times of the year. Skriabine and Schulz (1936), reporting the results ot their own research and that of other Russian workers, stated that in the free state, larvae were able to live for 90 days in water and faeces, but were never recovered from faeces which had been under snow. They also described the vertical distribution of larvae on herbage. In a study of longevity, Wetzel (1945), in Germany failed to recover larvae in May 1944, from plots of land, which had been contaminated in October 1943 and concluded that they were unable to survive the winter. Borchert and Timm (1949), however, recovered larvae in May 1949 from a pasture which was known to have been contaminated in October 1948 and had been vacant since December 1948. They were of the opinion however, that a severe winter would probably destroy most of the larvae, and the few which survived to the spring would be insufficient to cause any marked infection. Soliman (1952) made direct observations on the longevity of larvae out of doors in South England, during August and September. He used a variety of different plants grown in boxes and recorded the maximum longevity as five weeks.

The study of the bionomics of the free-living larvae of parasitic nematodes is beset by a number of difficulties. Under the controlled conditions of the laboratory it is possible to determine with accuracy the effects of different physical factors. This information is, however, of limited value by itself on account of the difficulty in relating it to naturally occurring situations, where the larvae are subjected not only to the effects of various physical factors all operating at the same

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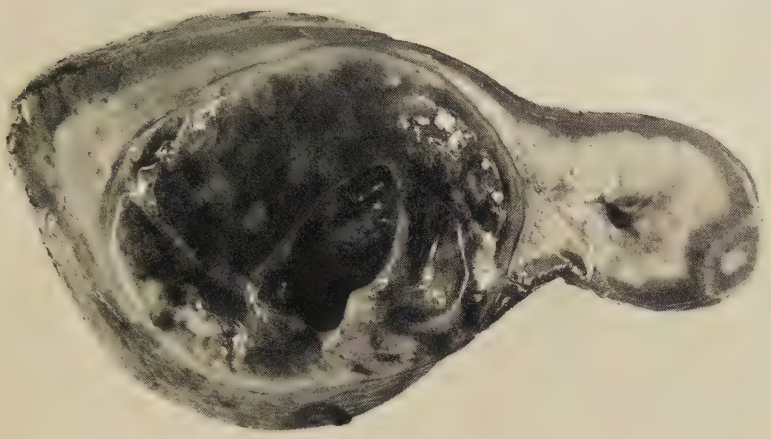
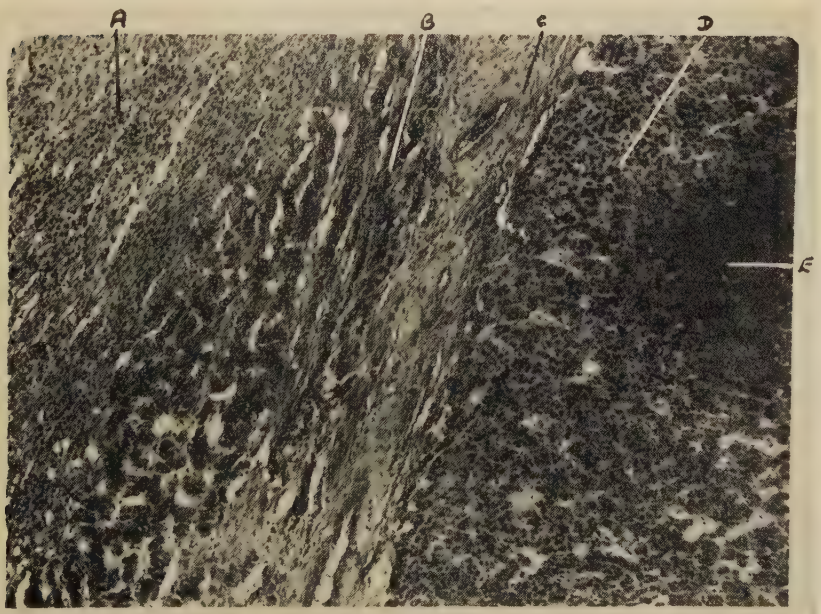
Fig. 1



Cut surface of left adrenal and tumour x 1½.

Fig. 2



Photomicrograph of cortex (A), medulla (B), tumour capsule (C), tumour (D), and haemorrhage in the tumour (E). Paraffin section ; stained H.E. x 170.

To face page 368

time, but also to biological factors. The larvae form part of an ecological unit and will almost certainly be affected by other living organisms within this unit.

Field observations, on the other hand, have their limitations. Their results are applicable only to the particular circumstances under which they are made and these may never be repeated in respect of climatic and other conditions. Moreover the many factors which operate at the same time make it impossible to assess the effects of any one, so that only tentative conclusions can be reached regarding the causes underlying what has been observed. It would seem best, therefore, to carry out observations both under natural conditions and in the laboratory, making use of the results of the one method in order to interpret those of the other: this has been done in the study reported in the present paper.

MATERIALS AND METHODS

Observations out of Doors

These observations were designed to assess the longevity, activity and rate of development at different times of the year. To avoid, as far as possible, contamination with free living nematodes and their larvae, all observations, except those on the vertical migration of the larvae, were carried out using herbage grown in boxes.

The boxes used, each measuring 28″ x 28″ x 4″, were made of galvanised iron, with a number of holes drilled in the bottom to allow for drainage. Each was divided into 49 small squares approximately 4″ x 4″, by means of strips of galvanised iron which projected 1/2″ above the soil surface. The soil was sterilised and formed a layer approximately 3½″ deep, below which was a layer of broken pot. The boxes were sown with 2 oz. of a pasture seeds mixture consisting of perennial ryegrass 40 per cent, chewings fescue, rough stalked meadow grass and brown top, just less than 20 per cent of each, white clover 2 per cent, and yarrow, a trace. When the herbage had grown for three months, it was clipped to a height of 1½″ and freshly passed faeces in 30 g. quantities were put in the centre of alternate squares. The faecal pellets were first thoroughly mixed together to obtain an even distribution of the larvae and the numbers present in two samples of 30 g were estimated at the start of each experiment.

The boxes were protected from birds by means of a wire netting cover. In the first experiment they were sampled at weekly intervals up to the 11th week, and once every two weeks thereafter. In subsequent experiments, samples were taken at weekly intervals throughout. In sampling, the faeces on a square were removed, the herbage was clipped to soil level, and the top half inch of soil cut away. Whenever it was apparent that most of the larvae had died off a fresh experiment was started.

Separation of larvae from the faeces was effected by the modified Baermann method, using a metal funnel and screen. The faeces were kept in the apparatus for 3 days. At intervals of 24 hours, 50 ml. of liquid were drawn off and the larvae in two samples of 25 ml. were counted under a dissecting microscope. The soil was treated in the same way except that it was left in the Baermann apparatus for 4 days and the first sample of 50 ml. was drawn off after 48 hours.

The herbage was cut into 2 in. lengths and washed in two pints of water which was allowed to stand for 24 hours and stirred at intervals. The washings were then passed through a coarse sieve into one or more round bottomed pint measures. After sedimenting overnight, the supernatant fluid was syphoned off and the residue centrifuged in conical tubes for 2 minutes at 2,000 r.p.m. The sediment was then examined microscopically and the larvae counted. This whole procedure was carried out three times with each sample of herbage, larvae frequently being recovered from the second washing although rarely from the third.

Some observations were also made on vertical migration for which purpose use was made of Italian ryegrass in 6″ pots which had been grown from seed for 3 months and was between 2½ to 3 inches high at the start of each experiment. Approximately 2,000 active third stage larvae, suspended in 20 ml. of water, were carefully pipetted over the soil surface and the pots were kept out of doors. Each day for one week the grass was cut from two pots in 4 lengths which, from the soil upwards, measured successively, ½″, ½″, 1 inch, ½″ to 1″. The larvae on the different lengths were recovered and enumerated as already described. The top ½″ of soil was also removed and larvae separated as before. The experiments were carried out at intervals between March and October and the grass was cut at various times of the day.

Laboratory Experiments

Laboratory experiments were carried out to assess the effects of temperature and humidity on the longevity of larvae in the faecal pellets, the effects of extreme desiccation and of temperatures below freezing point on longevity, and the effects of temperature on the rate of development.

It was considered desirable to carry out experiments on the effect of desiccation as the results of previous workers are not in agreement, and in no instance is there any record of the relative humidity at which desiccation took place.

The effect of temperature on the rate of development was carried out under conditions which compared more closely with those occurring naturally, than did the conditions that had been employed by previous workers.

The survival of free living larvae on wet and dry faeces at different temperatures. Freshly collected faeces were thoroughly mixed together and 30 g. put into each of a number of petri dishes which were divided into two groups A and B. In group A the faeces in closed petri dishes were stored in a covered pneumatic trough containing a small amount of water in the bottom and thus remained moist throughout the experiment. In group B, the faeces were allowed to dry before the petri dishes were closed and stored in a dry pneumatic trough. They were kept at three different temperature ranges 3 to 6°C, 12 to 22°C (room temperature) and 23 to 25°C. Samples of faeces were taken from each group at each temperature range, at weekly intervals for the first 16 weeks and subsequently at intervals of four weeks. The larvae were extracted from the faeces by means of the Baermann apparatus 100 ml. of liquid being drawn off on three consecutive days. The living larvae in two samples of 10ml. were enumerated, and the total number surviving in 30 g. of faeces was estimated. Any larvae which had migrated on to the petri dish were included in this final estimation.

The effect of extreme desiccation on longevity of first, second and third stage larvae. Approximately 100 larvae in 0.1 ml. of tap water were pipetted on to each of a number of glass slides which were kept at room temperature and humidity until only a thin film of water remained. Several slides were then put into each of three desiccators, in which relative humidities of 25 per cent, 50 per cent and 75 per cent respectively were obtained by placing sulphuric acid of known specific gravities in the bottoms of the desiccators and were checked by means of paper hygrometers. Some larvae were kept at a relative humidity of 100 per cent, as a control, to show that mortality was due to desiccation and others, kept at room humidity, served as a control to show that mortality of the larvae did not result from the presence of the acid in the desiccators.

At intervals of 24 hours, a slide was removed from each desiccator and 0.1 ml. of tap water pipetted on to the dried up larvae. The numbers of active larvae present at one hour and at 24 hours after wetting were counted. Preliminary observations had shown that there was some individual variation in the rate of recovery but by the end of 24 hours all living larvae were showing signs of activity.

The effect of freezing. The effect of temperatures below freezing point on the first, second and third stage larvae was determined as follows:— Approximately 300 larvae in 50 ml. of tap water were pipetted into a number of watch glasses which were placed in a refrigerator in a position where the temperature was 8°C. During the next two hours they were moved nearer to, and finally placed in the freezing unit, thus avoiding a sudden drop in temperature. Within a short time the larval culture was frozen solid. At intervals of 24 hours frozen larvae were removed to room temperature when they thawed out within 1 hour and some regained activity quickly whilst others took much longer. The numbers active were counted 1 hour and 24 hours after removal from the freezing unit. In between counts, the larvae were stored in the refrigerator at 8°C and control larvae were also kept at this temperature.

The effect of temperature on the rate of development. Most of the previous observations on the effect of temperature have been carried out using cultures of larvae in water, starting from the egg stage. Hatching however always takes place at the body temperature of the host either in the lungs or alimentary canal. In the present observations therefore faeces were collected from the rectum of an infected sheep and quickly placed in petri dishes in a constant temperature cabinet so that the rate of development of the larvae at 5, 10, 15, 20 and 25°C might be studied. There were slight fluctuations from the required temperature but these never exceeded one degree. The faeces were kept moist throughout the experiments in the manner described in an earlier part of this paper. To examine the larvae, samples of faeces were shaken in water in a petri dish when the larvae passed into the water almost immediately and were picked out by means of a pipette. They were then examined microscopically, some while still active, others after being made inactive by gentle heating.

RESULTS

The numbers of larvae recovered on alternate weeks from the faeces, herbage and soil of the boxes kept out of doors are recorded in Table 1. The vertical distributions on grass blades as indicated by

the numbers of larvae recovered from the different lengths of grass, is given in Table 2. The results of one experiment only are given as the pattern of vertical distribution was much the same in all of them. The results of the laboratory experiments are recorded in Tables 3 to 7.

TABLE 1

RECOVERY OF LIVING LARVAE OF *D. FILARIA* FROM FAECES, HERBAGE AND SOIL FROM GRASS BOXES KEPT OUT OF DOORS.

	Time in weeks		*No. of larvae recovered from:* 30 *g. Faeces*	*Herbage*	*Soil*
Expt. 1	0	(January 1st, 1952)	3,170		
	1		125	717	215
	3		0	275	12
	5		0	160	20
	7		42	140	0
	9		70	101	10
	11		20	282	0
	13		0	41	5
	15		0	26	0
	17		0	7	0
	19		0	6	0
	21		0	3	0
	23		0	2	0
	25	(June 23rd)	0	0	0
Expt. 2	0	(June 10th)	4,230		
	1		45	91	2
	3		7	17	1
	5		0	2	0
	7		0	1	0
	9	(August 12th)	0	0	0
Expt. 3	0	(August 5th)	3,760		
	1		2,325	131	91
	3		26	743	12
	5		0	594	7
	7		9	91	0
	9		7	40	0
	11		3	27	0
	13		0	3	0
	15		0	1	0
	17	(December 2nd)	0	0	0
Expt. 4	0	(November 18th)	5,224		
	1		3,064	310	500
	3		1,104	75	24
	5		290	25	16
	7		164	57	8
	9		142	65	5
	11		124	28	3
	13		70	76	0
	15		14	42	3
	17		8	14	2
	19		0	0	2
	21	(April 14th)	0	0	0

The longevity of the larvae varied according to the time of year. The maximum was 23 weeks (January 1st to June 23rd); the minimum 7 weeks (June 10th to August 5th) (Table 1). It would appear

TABLE 2

VERTICAL MIGRATION OF THIRD STAGE LARVAE UP BLADES OF GRASS.

Position on grass blade	*No. of larvae recovered*
Top ½″ — 1″	0
Middle 1″	2
Middle ½″	37
Bottom ½″	318

that the conditions prevailing during this latter period were least favourable for survival, for not only was the maximum longevity reduced, but there was also a very heavy initial mortality, greater than occurred at any other time of the year. During June and July temperatures were high and there was little rainfall (Table 8); as a result the faecal pellets dried out very rapidly after being put on the herbage. It was assumed that the heavy initial mortality resulted from the rapid drying out to which the larvae must have been subjected. This assumption was supported by the laboratory experi-

TABLE 3

LONGEVITY OF THE LARVAE OF *D. FILARIA* ON WET FAECES.

Weeks	*Percentage of living larvae surviving*		
	3–6°C	14–22°C	23–25°C
0	100	100	100
4	22	14	13
8	37	9	2
12	19	4	<1
16	11	2	
20	4	<1	
24	5	<1	
28	2	<1	
32	4	<1	
36	2	0	
40	<1	0	
44	<1		
48	<1		
52	0		
56	0		

Although faeces were sampled at weekly intervals for the first 16 weeks, only the percentage of living larvae regained every 4th week are recorded on Table 3.

ments which showed that the mortality among larvae in the dried out faeces (Table 4) was much greater than that in the wet ones (Table 3). The maximum period of survival was also greatly reduced

TABLE 4

LONGEVITY OF THE LARVAE OF *D. FILARIA* ON DRIED FAECES.

Weeks	*Percentage of living larvae surviving*		
	3–6°C	14–22°C	23–25°C
0	100	100	100
1	22	19	10
2	21	18	5
3	18	2	<1
4	13	1	<1
5	8	<1	0
6	5	<1	0
7	3	<1	
8	2	<1	
9	2	<1	
10	1	0	
11	<1	0	
12	<1		
13	<1		
14	<1		
15	0		
16	0		

TABLE 5

THE EFFECT OF DESICCATION ON SURVIVAL OF LARVAE OF *D. FILARIA*.

Days of desiccation	*Percentage active 1 hr. after wetting*				*Percentage active 24 hrs after wetting*				*R.H. of room*	*Temperature °F*	
	Relative humidity				*Relative humidity*						
	25%	50%	75%	*Room* %	25%	50%	75%	*Room* %		*Min.*	*Max.*
				First stage larvae							
0	100	100	100	100	100	100	100	100	48	54	61
1	0	0	0	0	0	0	0	0	46	52	60.5
				Second stage larvae							
0	100	100	100	100	100	100	100	100	47	54	61
1	0	4	3	0	1	30	53	1	39	54.2	61
2	0	0	0	0	0	0	14	0	46	54	61
3	0	0	0	0	0	0	11	0	54	55	62
4	0	0	0	0	0	0	0	0	54	56.5	62
				Third stage larvae							
0	100	100	100	100	100	100	100	100	58	54	61
1	0	0	17	11	0	4	42	38	51	53.5	61
2	0	0	8	7	0	0	19	15	64	54	61
3	0	0	0	0	0	0	14	12	67	54	61
4	0	0	0	0	0	0	4	4	71	56.5	63
5	0	0	0	0	0	0	0	0	72	57.5	60

in the dry faeces. Further confirmatory evidence was seen from the experiments on the effects of extreme desiccation. From Table 5 it can be seen that third stage larvae are the most resistant, a few surviving for 4 days at a relative humidity of 75 per cent, but mortality being more rapid under drier conditions. The second stage larvae were almost as resistant as the third stage, but first stage larvae succumbed readily.

The effect of high temperatures alone would be expected to reduce longevity, owing to increased activity and metabolism of the larvae and more rapid utilisation of food reserves. That such a reduction did take place can be seen from Table 3, which shows that larvae on wet faeces survived for shorter periods at high than at low temperatures. It should be noted, however, that at the high temperatures, fungi and bacteri grew rapidly on the wet faeces and these organisms may have accelerated the rate of mortality of the larvae. Deschiens (1939) has in fact shown that predatory hypomycetes can destroy *D. filaria* larvae.

Out of doors, larvae survived well when temperatures were high and an adequate amount of moisture was available (Table 1): such conditions were prevalent during August (Table 8) when survival of the larvae was much better than during the two previous months. During the winter months temperatures were frequently below

TABLE 6

LONGEVITY OF THE LARVAE OF *D. FILARIA* AT TEMPERATURE BELOW FREEZING POINT.

Period frozen (Days)	*Percentage active 1 hr. after removal from freezer*			*Percentage alive and active 24 hrs. after removal from freezer*			*Control Percentage alive*			*Temp. °C.*	
	1st	*2nd Stage*	*3rd*	*1st*	*2nd Stage*	*3rd*	*1st*	*2nd Stage*	*3rd*	*Min.*	*Max.*
0	100	100	100	100	100	100		100			
1	60	56	40	68	64	100		100		−1.1	−7.6
2	10	8	10	57	62	89		100		−2.1	−7.6
3	0	1	1	46	60	86		100		−2.1	−7.2
4	0	0	1	33	44	82		100		−2.4	−7.2
5		0	1	21	34	80		100		−2.3	−7.2
6			0	17	30	78		100		−2.3	−7.4
7			0	14	28	73		100		−2.6	−7.8
8				9	26	59		100		−2.7	−7.2
9				5	22	49		100		−2.7	−7.4
10				1	14	32		100		−2.3	−7.2
11				<1	8	28		100		−2.6	−7.4
12				<1	4	21		100		−2.6	−7.3
13				0	0	18		100		−2.6	−7.3
14				0	0	15		100		−2.7	−7.2
15						15		100		−2.7	−7.4
16						13		100		−2.5	−7.6
17						10		100		−2.3	−7.6
18						7		100		−2.3	−7.4
19						2		100		−2.6	−7.3
20						0		100		−2.6	−7.4

freezing point (Table 8) yet considerable numbers of larvae were able to survive (Table 1). A surprising degree of resistance to temperatures below freezing was also shown by larvae in the laboratory experiments (Table 6). There was no mortality amongst third stage larvae after being frozen continuously for 24 hours and some survived for a maximum of 19 days. First and second stage larvae were less resistant, but some survived for 12 days.

The activity of the larvae is well marked. In the outdoor observations many were recovered from the herbage (Table 1), indicating that they had either migrated, or had been washed away, from the faeces. That they can actively migrate was readily demonstrated in the laboratory when faeces were kept in petri dishes and larvae were seen to move to the sides and tops of the containers provided that an adequate amount of moisture was present. Under dry conditions this migration rarely occurred. Larvae which reached the herbage were seen to be capable of migrating up the blades of grass into a position where they could be ingested by grazing sheep, and most of them were recovered from the lowest $\frac{1}{2}''$ of the blades (Table 2). At certain times of the year, notably in November and December, large numbers were recovered from the faeces and far fewer from the herbage (Table 1). It is probable that the low temperatures then prevailing had curtailed their powers of migration. Such decreased activity was found to occur in the laboratory when larvae had been kept at a low temperature in the refrigerator.

As expected, the rate of development was found to vary at different times of the year. In the outdoor observations started on November 18th, 1952, a few third stage larvae were first seen at the end of six weeks. There was an appreciable degree of individual variation at this time of the year but by the end of the ninth week most of the larvae had reached the third stage. It was rather surprising to find that some third stage larvae were present in faeces exposed out of doors on January 1st, 1952, after only 4 weeks, and all were at this stage after five weeks though the temperature during this period was often below freezing point. The rate of development during June and August was much more rapid and all the larvae were at the third stage within a week. The rates of development at

TABLE 7

EFFECT OF TEMPERATURE ON THE RATE OF DEVELOPMENT OF LARVAE OF *D. FILARIA*.

Temperature °C	*Minimum time in days to develop to*		
	2nd stage	*3rd stage*	*Infective stage*
5	9	17	20
10	2	4	8
15	2	4	6
20	2	4	5
25	1	2	5

known temperatures (Table 7) are in keeping with what was seen in the outdoor observations. It is interesting to note that development will proceed quite rapidly at temperatures as low as 5°C.

TABLE 8
MEAN MONTHLY MINIMUM AND MAXIMUM TEMPERATURES AND TOTAL MONTHLY RAINFALL FROM DECEMBER 1951 TO FEBRUARY 1953.

Month	*Temperature °F*		*Total monthly rainfall (inches)*
	Mean monthly minimum	*Mean monthly maximum*	
1951 December ..	35	50	1.46
1952 January	29	44	1.59
February ..	31	45	0.81
March	37	52	2.2
April	39	59	1.78
May	45	68	1.21
June	44	69	1.12
July	52	74	0.55
August	52	71	3.12
September ..	42	61	3.18
October	39	55	2.74
November ..	31	45	1.94
December ..	30	44	2.14
1953 January	30	38	0.76
February ..	33	46	1.53

DISCUSSION

In correlating the effects of the various factors on the larvae, it can be seen that certain sets of conditions are more favourable to them than others. In general it would appear that larvae will have most chance of reaching the final host and thereby completing the life cycle, under warm moist conditions, when mortality is reasonably low, the rate of development is rapid and they are very active. Cool moist conditions also favour survival but development is slow and activity is reduced. A combination of high temperatures and dryness are least favourable and result in a high mortality. In general, the most favourable conditions for infection of the final host to take

place are likely to occur in the spring and autumn and also during the summer on pastures which are wet either as a result of heavy rainfall or other causes. In a dry summer however, the chances of infection occurring will be greatly reduced, as also during the winter. Larvae were able to withstand the winter conditions prevailing in South England, but since only a few survived until the following spring it does not seem likely that outbreaks of parasitic bronchitis at this time of the year are due to the small numbers of larvae which have overwintered. It seems more likely that the pastures have been freshly contaminated by carrier animals.

CONCLUSIONS

Observations on sheep faeces out of doors, and on the surrounding herbage and soil, indicated that the period of survival of the larvae of *D. filaria* varied from season to season. They persisted for 23 weeks from January to June, but for only 7 weeks from June to August.

Many larvae passed out from the faeces on to the herbage. They were capable of migrating from the soil surface up to within 1″ of the tip of grass blades 3″ high.

The rate of development varied with the time of year; during November and December at least 6 weeks were required for larvae to develop to the third stage, but less than one week during June and August.

In the laboratory, larvae survived best in wet faeces at low temperatures. Under such conditions the maximum period of survival was 48 weeks as compared with only 14 weeks on dry faeces.

Extreme desiccation resulted in rapid mortality. Third stage larvae, which were the most resistant, survived for a maximum of 4 days at a relative humidity of 75 per cent but died out more rapidly under drier conditions.

Marked resistance was shown to temperatures below freezing point, some third stage larvae surviving for 19 days.

The rate of development was slowed down as the temperature was lowered. At 5°C, infective larvae were first observed after 20 days, whereas at 25°C this stage was reached in 5 days.

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